Remarks

Finality of Restriction Requirement

The restriction requirement mailed June 30, 2004 has been made final. Thus, in an earnest effort to advance the prosecution of this case, Applicants have canceled without prejudice non-elected claims 16 – 22, 24, and 26 – 30. Applicants have also canceled claim 31 without prejudice. Applicants reserve the right to file one or more divisional applications drawn to the canceled subject matter.

Claims 1-15, 23 and 25 are pending. Claims 1-3 and 9-12 have been amended. Amendments in Claims 1, 9, 10 and 12 regarding serine phosphorylation are made to more distinctly identify Applicants invention. Support for these amendments can be found on page 5 lines 1-3 and pages 20 line 8 through 21 line 2. Claims 2, 3, 11 and 12 have been amended as described elsewhere herein. New claim 32 has been added. Support for the new claim can be found throughout the specification and original claims. No new matter has been added by way of amendment. Reconsideration is respectfully requested in light of these amendments and the following remarks.

Specification/Informalities

The title has been changed, at the request of the Examiner, to more accurately describe the claimed invention.

The paragraph beginning on line 4 of page 1 has been amended to correct the NIH grant number.

35 U.S.C. § 112, Second Paragraph Claim Rejections

The Examiner has rejected Claims 1 -15, 23, 25 and 31 as being indefinite for failing to particularly point out and distinctly claim the subject matter which Applicants regard as the invention.

[a] The Examiner has rejected claims 1 (claims 2-15, 23 and 25 dependent therefrom) and 31 on the grounds that they are incomplete as the claims fail to set forth any method steps for determining whether Dab1 is phosphorylated (claim 1) or for determining the amount of phosphorylated Dab1 and total amount of Dab1 in a biological sample (claim 31). This rejection is moot with respect to claim 31, which has been

canceled. This rejection is respectfully traversed with respect to the remaining pending claims for the following reasons.

The present invention is directed to the discovery that Dab1 is specifically phosphorylated by Cdk5. There are multiple methods well known in the art for determining whether a protein is phosphorylated at a particular site. They include, but are not limited to: 1. Immunoprecipitation with antibody that binds only the phosphorylated protein. 2. Partial purification of a sample and then analyzing its phosphorylation. 3. Identification of unique peptides by mass spectrometry. 4. Immunoprecipitation of both phosphorylated and unphosphorylated forms of the protein and then analyzing phosphorylation. 5.Using a phosphor-antibody to western blot a particular protein. 6. Phosphopeptide mapping of proteins. 7. In vitro kinase assays. 8. Immunohistochemistry and 9. ELISA assays. Applicants teach and use multiple methods for detecting phosphorylation of a protein including phosphopeptide mapping (page 18 lines 23-29, page 20 lines 3-7) and in vitro kinase assays (page 18 lines 16-22, page 20 lines 18 through page 21 line 2). Therefore, claim 1 should not be limited to a particular method for determining phosphorylation since there are numerous methods used routinely by those in the field to measure phoshorylation.

[b] The Examiner has rejected claims 1 (claims 3-6, 11 and 13-14 dependent therefrom), 2, 7-10, 12, 15, 23, 25 and 31 for being indefinite in the recitation of "Cdk5", "Cdk5 activity" and "Dab1".

The Examiner asserts that it is unclear as to the polypeptides that are meant to be encompassed by the term "Cdk5" and "Dab1". The Examiner further asserts that the definition of "Cdk5 activity" encompasses the activity of any serine/threonine kinase and it is unclear as to how one distinguishes a "Cdk5 activity" from the activity of other serine/threonine kinases.

Applicants respectfully disagree. The terms Cdk5 and Dab1 are familiar terms well known to those of skill in the art and have a clearly understood meaning. Cdk5 is distinguished from other cyclin dependent kinases by the fact that it is not involved in cell cycle regulation and its active form is found only in differentiated neurons of the developing and mature brain (Introduction on page 1 lines 12 - 24 and references cited

therein, Lew et al., J Biol Chem 267:13383-13390, 1992; Meyerson et al., EMBO J 11:2909-2917, 1992). It has also been shown that Cdks other than Cdk5 are activated by cyclins whereas Cdk5 is activated by p35 which is completely different from known cyclins. Applicants have shown that Cdk5 is distinguishable from other cyclin dependent kinases. Therefore, "Cdk5 activity" is distinct from the activity of other serine/threonine kinases.

Numerous references are cited throughout the specification, including Howell et al., Nature 389:733-737, 1997 (page 13 line 27 and page 25 line 10 of the specification) which distinguish Dab1 from various species. Dab2 protein has the closest homology with Dab1. However, the homology between Dab1 and Dab2 begins to break down after the first 170 amino acids. In particular, the region of Dab1 that is phosphorylated by Cdk5 is not conserved within the Dab family.

Applicants again stress that the present invention is not based on the discovery of the Dab1 or Cdk5 proteins or their general activities which were well known in the art. The present invention is instead based on the discovery that Dab1 is specifically phosphorylated by Cdk5. Applicants have shown that the terms "Cdk5", "Dab1" and "Cdk5 activity" are well known in the prior art. Therefore, the present invention should be directed to a broad genus of Cdk5 and Dab1 proteins. Applicants request reconsideration and withdrawal of these rejections on this basis.

[c] The Examiner rejected claims 1 (claims 2, 4-9, 11-15, 23 and 25 dependent therefrom), 10 (claims 12-15 dependent therefrom), and 31 as being indefinite in the recitation of "a candidate sequence preferred by Cdk5 activity". Applicants respectfully disagree.

Claims 1, 9,10 and 12 have been amended to show that a serine within a candidate sequence is the site of Cdk5 phosphorylation. Page 5, line 1 of the specification specifically defines a "candidate sequence" as a sequence of amino acids which contains a serine followed by a proline in the +1 position and a lysine in the +3 position, the serine being a preferred site for Cdk5 activity (Songyang et al., Mol Cell Biol, 16:6486-6493, 1996). Songyang et al. teach that this sequence is a distinct optimal peptide substrate for the Cdk5 kinase. Further, on page 20 lines 8-18, applicants predicted

murine Dab1 serines 491 and 515 to be cdk5 phosphorylation sites based on sequence analysis and then conducted experiments to show their prediction was true. Claim 31 has been canceled, rendering this rejection moot with respect to this claim. Reconsideration and withdrawal of this rejection as applied to the remaining pending claims is respectfully requested.

[d] The Examiner rejected claim 2 as being confusing in the recitation of "the Cdk5 amino acids consisting of serine 491 and 515". Applicants apologize for this erroneous reference and have revised the claim to correctly refer to Dab1 which is consistent with the teachings throughout the specification. Reconsideration is respectfully requested.

[e] The Examiner rejected claims 2 and 12 (claim 25 dependent therefrom) as being unclear in the recitation of serine 491 and 515 as there is no reference sequence recited in the claims to identify serine residues that are considered to be at positions 491 and 515. Applicants have amended the claims to include the appropriate sequence listings that contain the murine Dab1 491 and 515 serine phosphorylation sites. Support for the claim amendments can be found on page 20 lines 13 – 18. Reconsideration is respectfully requested.

[f] The Examiner rejected claim 3 as being confusing as the sequences of SEQ ID NO:1 and 2 are recited as "tryptic peptides"/ however, the method of claim 1 recites "wherein phosphorylation of Dab1… …indicates the presence of active Cdk5." The Examiner contends that based on the definition of Dab1, it does not appear that either of SEQ ID NO:1 or 2 is considered to be a Dab1 polypeptide. Applicants have amended Claim 3 and deleted "tryptic peptides" from the claim. Reconsideration is respectfully requested.

[g] The Examiner rejected claims 4-6 as being unclear in the recitation of "derived from". Applicants respectfully disagree.

Claims need only be as precise as the subject matter permits to comply with the definiteness requirement of 35 USC § 112, second paragraph. *Verve, LLC v. Crane Cams*, Slip No. 01-1417 (Fed. Cir Nov. 14, 2002). The Cambridge International Dictionary of English definition for derived is: to get or obtain something from something else. The

term "derived from" is well known in the art and is intended to mean tissues, blood etc. isolated directly from a subject as well as other substances, such as cultured cell lines, genes, proteins etc., which are derived from the directly isolated tissues. Applicants feel that the term "isolated from" does not adequately reflect the scope of samples to which the claimed method can be applied. Further, Applicants point out that the phrase "derived from" in clearly not inherently indefinite since it appears in the granted claims of over 3000 patents issued in the year 2004. Applicants respectfully submit that the phrase "derived from" is sufficiently definite in the context in which it is used in the pending claims. Reconsideration and withdrawal of this rejection is therefore respectfully requested.

[h] The Examiner rejected claim 11 as being confusing in the recitation of "contains a phosphate group on serine 491" as SEQ ID NO:3 has only 14 amino acids and it is unclear as to how a 14-amino acid sequence has a serine at position 491. Claim 11 has been amended to particularly point out that the eighth amino acid of SEQ ID NO:1 is phosphorylated. Support for such amendment can be found on page 3 lines 25 – 29. Applicants respectfully request reconsideration of this rejection.

[i] The Examiner rejected claim 31 as being unclear in the recitation of "wherein the proportion of Dab1 which is phosphorylated on said candidate sequence represents a quantitative measure of the level of Cdk5 activity. The Examiner contends that it is unclear from the claims and the specification as to how one converts "the proportion of Dab1 which is phosphorylated" into a "quantitative measure of the level of Cdk5 activity."

Applicants respectfully disagree. However, in an attempt to further prosecution, Applicants have canceled claim 31 without prejudice, thus rendering this rejection moot.

35 USC § 101 Claim Rejection

Claims 1-15, 23, 25 and 31 are rejected under 35 U.S.C. 101 because the claimed invention is not supported by either a specific and substantial asserted utility or well-established utility. The Examiner asserts that there is no evidence in the specification that links Cdk5 activity or level with a specific disease or disorder, particularly a neurological disorder, such that one could use the methods for detection of a particular disease state or

disorder. Further, the Examiner asserts that the specification fails to provide guidance as to a specific neurological disorder that can be detected by increased Cdk5 activity or a correlation of a specific neurological disorder with increased Cdk5 activity.

Applicants respectfully disagree. Patrick et al., Nature 402:615 – 622, which is incorporated within the specification (page 1 line 22, page 2 line 26, page 11 line 10, and page 27 line 6) specifically states that Cdk5 is required for proper development of the mammalian central nervous system. To be activated, Cdk5 has to associate with its regulatory subunit, p35. They found that p25, a truncated form of p35, accumulates in neurons in the brains of patients with Alzheimer's disease. *This accumulation correlates with an increase in Cdk5 kinase activity.* Furthermore, Nguyen et al. Neuron 30:135-147, 2001 shows that unregulated Cdk5 activity is implicated in the pathology of amyotrophic lateral sclerosis (page 2 lines 24-27 of the specification).

Ohshima et al., Proc Natl Acad Sci USA 93:11173-11178, 1996 incorporated into the specification by reference (page 2 lines 11-12 and 22, page 11 line 8, page 13 line 30, page 25 line 12) shows that Cdk5 -/- mice exhibit characteristics seen in several neurodegenerative disorders, such as motor neuron disease, progressive supranuclear palsy, Lewy body disease and chemical intoxication by neurotoxic substances such as aluminum, $\beta_2\beta$-iminodiproprionitrile and 2,5-hexanedione. Thus, the lack of Cdk5 activity is associated with these diseases.

Due to the fact that Cdk5 activity is tightly controlled by its regulator, p35, Cdk5 activity is difficult to determine based on levels of Cdk5 present. Furthermore, a substrate which is selectively phosphorylated by Cdk5 had heretofore not been identified. The present invention shows that phosphorylation of Dab1 on serines 491 and 515 is Cdk5 dependent; thus, providing a method to quantitatively measure Cdk5 activity. An assay showing quantitative levels of Cdk5 activity by detecting serines within a sequence preferred by Cdk5 activity in Dab1 provides a very useful tool for detecting neurological disorders, including Alzheimer's disease.

A separate patentable utility for the methods of the present invention is to identify compounds that regulate Cdk5 activity. Since an increase of Cdk5 activity has been

shown to be associated with Alzheimer's disease, compounds that inhibit Cdk5 activity may prove to be useful therapeutics to treat this disease.

Numerous studies link Cdk5 activity to neurological diseases, in particular, increased Cdk5 activity has been shown to be linked to Alzheimer's disease. Therefore, a method to detect the disease and a method to identify therapeutic compounds for the disease are credible utilities. Although the detection of Cdk5 activity by detecting Dab1 phosphorylation on a serine within a candidate sequence preferred by Cdk5 activity has multiple utilities, a showing of one credible utility is sufficient to meet the requirement of 35 U.S.C. § 101. See, *Raytheon v. Roper,* 724 F.2d 951, 958 (Fed. Cir. 1983), *cert denied*, 469 U.S. 835 (1984) ("When a properly claimed invention meets at least one stated objective, utility under 35 U.S.C. 101 is clearly shown."). Accordingly, the claimed invention clearly has at least one specific and substantial utility. On this basis Applicants request that this rejection be reconsidered and withdrawn.

The Examiner has rejected claims 1-15 ,23, 25 and 31 under 35 U.S.C. §112, first paragraph because, according to the Examiner, the claimed invention is not supported by either a credible asserted utility or a well established utility and, therefore, one skilled in the art would not know how to use the claimed invention.

A lack of utility rejection under 35 U.S.C. §101 also creates a rejection under 35 U.S.C. §112, first paragraph. See *In re Brana,* 51 F.3d 1560 (Fed. Cir. 1995). However, a lack of utility rejection under 35 U.S.C. §112, first paragraph should not be imposed or maintained unless an appropriate basis exists for imposing the lack of utility rejection under 35 U.S.C. §101. See MPEP 2107.01(IV). The remarks in the section immediately above point out the utility of the claimed invention under 35 U.S.C. §101 as disclosed in the specification. Since the invention is useful, the rejection under 35 U.S.C. §112, first paragraph, enablement should be reconsidered and withdrawn.

35 USC § 112, First Paragraph Claim Rejections

The Examiner has rejected Claims 1-15, 23, 25 and 31 under 35 USC 112, first paragraph, as failing to comply with the written description requirement. The Examiner asserts that the claims contain subject matter that was not described in the specification in

such a way as to reasonably convey to one skilled in the relevant art that the inventors, at the time the application was filed had possession of the claimed invention. Claims 1-15 are drawn to methods for detecting the activity of a genus of Cdk5 polypeptides by determining whether a genus of Dab1 polypeptides has been phosphorylated, and optionally wherein the method uses a genus of antibodies that bind to phosphorylated Dab1 or the method is used for detecting neurological disorders. Claims 23 and 25 are drawn to methods for detecting neurological disorders using the method of claims 1 or 12. Claim 31 is drawn to a method for quantitating the level of activity of a genus of cdk5 polypeptides by determining the amount of a genus of phosphorylated Dab1 polypeptides. The Examiner asserts that the written description requirement for a claimed genus is not satisfied due to a lack of sufficient description of a representative number of species for Cdk5, Dab1 and recited antibodies.

As the Examiner points out, for claims drawn to a genus, MPEP §2163 states the written description requirement for a claimed genus may be satisfied through sufficient description of a representative number of species by actual reduction to practice, reduction to drawing, or by disclosure of relevant, identifying characteristics, i.e., structure or other physical and/or chemical properties, by functional characteristics coupled with a known or disclosed correlation between function and structure, or by a combination of such identifying characteristics, sufficient to show the applicant was in possession of the claimed genus. A representative number of species means that the species which are adequately described are representative of the entire genus. Thus, when there is substantial variation within the genus, one must describe a sufficient variety of species to reflect the variation within the genus.

Regarding the genus of Cdk5 and Dab1polypeptides, the Examiner acknowledges that the specification discloses three representative species of Cdk5 polypeptides and two representative species of Dab1 polypeptides. The Examiner asserts that the genera of Cdk5 and Dab1 polypeptides encompass widely variant species with respect to structure and that Applicants fail to describe a sufficient number of species of the genus of Cdk5 and Dab1 polypeptides, which encompass mutants and variants of known Cdk5 and Dab1

polypeptides and wild type and mutant sequences from any organism – including those that have yet to be identified. Applicants respectfully disagree.

The present invention is directed to methods for detecting Cdk5 activity. This has been difficult in the past because Cdk5 activity is tightly controlled by its regulator, p35, making it difficult to determine activity based on levels of Cdk5 present. The discovery that Dab1 is specifically phosphorylated on seine within a preferred candidate sequence by Cdk5 is the basis for the present invention. The invention is not based on the novelty or nonobviousness of Cdk5 or Dab1, but rather on the relationship between the two. The terms Cdk5 and Dab1 have distinct meanings well known by those skilled in the art. Members of each respective family have common structure, characteristics and activities which form the basis of their classification as a Cdk5 or a Dab1 protein. In view of the shared structural and functional characteristics of members of the Cdk5 and Dab1 families, one of skill in the art would have every reason to expect that the discovery of a specific phosphorylation event that forms the heart of this invention would be shared by other members of this family. In view of this rationale expectation the Examiner cannot simply conclude that this method will not work with other Cdk5-Dab1 combinations but rather must provide some rational explanation to support such a conclusion. Thus far no reason has been given for the skilled artisan to expect that a Cdk5 or Dab1 protein from another species or mutants and variants of a known protein will not have those characteristics needed for the phosphorylation of Dab1 on a serine within a preferred candidate sequence.

To satisfy the written description requirement it is not necessary that the application identify each and every species of the genus, but only to the extent that one having ordinary skill in the pertinent art would recognize from the disclosure that applicants invented the claimed subject matter. Identifying characteristics, i.e., structure and other physical and chemical properties of Cdk5 and Dab1 polypeptides are well known. Furthermore, procedures are well known in the art for identifying whether or not polypeptides, whether they be novel or mutants and variants of known Cdk5 or Dab1 polypeptides, possess these characteristics. Withdrawl of this rejection is respectfully requested.

Regarding the genus of recited antibodies, the Examiner asserts that the recited genus of antibodies encompasses species that are widely variant. Given that the specification discloses only a single representative species of the genus of recited antibodies, the specification fails to sufficiently describe the claimed invention in such full, clear, concise and exact terms that a skilled artisan would recognize that applicant was in possession of the claimed invention. Applicants respectfully disagree.

A Cdk5 preferred site within any protein is taught. A "candidate sequence" is defined as a sequence of amino acids which contains a serine followed by a proline in +1 position and a lysine in +3 position, the serine being a preferred site for Cdk5 activity (Songyang et al., Mol Cell Biol, 16:6486-6493, 1996) (page 5 lines 1-3 of the specification). Songyang et al. teach that this sequence is a distinct optimal peptide substrate for the Cdk5 kinase. One of skill in the art is capable of determining if a Dab1 protein contains a candidate sequence and then determining the sequence surrounding this site so that peptides useful for generating antibodies may be synthesized. Generation of antibodies to a given peptide sequence is well known in the art (page 5 line 20 through page 6 line 7). Therefore, it is not necessary for applicants to specifically teach each and every species in the genus. Reconsideration and withdrawal of this rejection is therefore respectfully requested.

Claims 1-15, 23, 25 and 31 are rejected under 35 USC 112, first paragraph because the specification does not reasonably provide enablement for the broad scope of the claimed methods. According to the Examiner, the specification does not enable any person skilled in the art to which it pertains, or with which it is most nearly connected, to make and/or use the invention commensurate in scope with the claims.

Regarding claims 1-15, the Examiner asserts that the claims are so broad as to encompass a method for detecting any Cdk5 activity in a biological sample by determining whether any Dab1 protein is phosphorylated on any sequence that is "preferred" by Cdk5 by any method or technique for detecting Dab1 phosphorylation. The Examiner asserts that claims 23 and 25 are so broad as to encompass methods of detecting any neurological disease by measuring an increase in Cdk5 activity. The Examiner asserts that claim 31 is so broad as to encompass a method for quantitating

Cdk5 activity in a biological sample by determining the amount of Dab1 that is phosphorylated on a sequence that is "preferred" by Cdk5, wherein the proportion of phosphorylated Dab1 to the total amount of Dab1 represents a quantitative measure of Cdk5 activity. The Examiner asserts that the broad scope of claimed methods is not commensurate with the enablement provided by the disclosure with regard to the extremely large number of Cdk5 polypeptides, Dab1 polypeptides and Cdk5 "preferred" sequences thereof, methods/techniques for detecting phosphorylation and quantitatively measuring Cdk5 activity.

Sufficient guidance and working examples

In regard to the scope of claims 1-15 the Examiner asserts that the specification provides only a single working example. The Examiner asserts the specification fails to provide guidance as to whether Cdk5 from all sources has the ability to phosphorylate Dab1 from a corresponding source, fails to teach those candidate sequences that are "preferred" by Cdk5 from any source, and further fails to provide guidance regarding all methods that can be used to detect Cdk5 phosphorylation of Dab1. Applicants respectfully disagree.

Little guidance is need to practice the invention as claimed because the components, Cdk5 and Dab1, are well known in the art, a preferred candidate sequence is well known and the methods for identifying a preferred candidate sequence within a protein are well known. Furthermore, methods for detecting phosphorylation of a protein at a specific site and the generation of reagents to do so are familiar techniques to one skilled in the art. There is no reason to believe that Cdk5 from any source will not phosphorylate Dab1 on a preferred candidate sequence. Applicants respectfully request that the Examiner provide evidence as to why one of ordinary skill in the art would not expect any Cdk5 to phosphorylate any Dab1 on a serine within the candidate sequence taught, and why the well known methods for detecting phosphorylation would not be expected to work.

In regard to the scope of claims 23, 25 and 31 the Examiner asserts that the specification fails to provide even a single working example of the methods claimed and

fails to provide any guidance regarding those neurological disorders that can be detected by measuring an increase in Cdk5 activity.

Applicants respectfully disagree. Claim 31 has been canceled, therefore rendering this rejection moot. Claim 23 depends from claim 1 and claim 25 depends from claim 12. Claims 1 and 12 both describe methods for detecting Cdk5 activity. As stated previously, increased Cdk5 activity has been associated with Alzheimer's disease (Patrick et al., Nature 402: 615 – 622), (page 1 line 22, page 2 line 26, page 11 line 10, and page 27 line 6 of the specification) and amyotrophic lateral sclerosis (Nguyen et al. Neuron 30:135-147), (page 2 lines 24-27 of the specification).

The high degree of predictability in the art

The Examiner asserts that the specification fails to provide guidance regarding the relationship of Cdk5 and Dab1 proteins from other sources and that it is highly unpredictable as to whether Cdk5 phosphorylates all Dab1 proteins, including those Dab1 proteins having sequences that vary from those that are disclosed in the specification. The Examiner further states that it is possible that Dab1 from other sources does not comprise a Cdk5 "preferred" sequence or that the antibody generated against SEQ ID NO:3 would not recognize phosphorylated Dab1 from other sources, or would recognize a phosphorylated form of Dab1 that is phosphorylated by a kinase other than Cdk5. The Examiner asserts that the ability to detect phosphorylation of a protein by a specific kinase by any method is highly unpredictable.

Applicants respectfully disagree. Cdk5 and Dab1 proteins are well known in the art. They are classified according to certain functional characteristics. Furthermore, a "candidate sequence" is defined by Songyang et al., Mol Cell Biol, 16:6486-6493, 1996. On page 19 lines 20 through page 21 line 2 of the specification, Applicants describe an assay for determining whether or not a protein would contain a Cdk5 phosphorylation site. Therefore, a person of skill in the art is capable of sequencing any Dab1 protein to determine if it contains a Cdk5 preferred candidate sequence. A person skilled in the art is then capable of using known techniques to determine if that site is phosphorylated. Such techniques are well known in the art.

Regarding claims 23 and 25, the Examiner asserts that it is highly unpredictable as to whether an increased level of Cdk5 activity is related to any neurological disorder. Applicants respectfully disagree. As shown previously, Patrick et al., Nature 402:615 – 622, (page 1 line 22, page 2 line 26, page 11 line 10, and page 27 line 6 of the specification) shows a correlation between increased Cdk5 activity and Alzheimer's disease and Nguyen et al. Neuron 30:135-147, 2001 (page 2 lines 24-27 of the specification) shows that unregulated Cdk5 activity is implicated in the pathology of amyotrophic lateral sclerosis. There are additional references (Appendix A) that show a correlation between increased Cdk5 activity and neurological disorders, Green et al, Neurochem Int., Oct; 31 (4):617-23 (1997) correlates increased Cdk5 activity with events associated with neuronal response to ischemic injury, Borghi et al., Neurology, Feb 26;58(4):589-92 (2002) show that increased Cdk5 is related to neurofibrillary pathology in progressive supranuclear palsy, and Bu et al., J Neurosci. Aug 1;22(15):6515-25 (2002) show that increased Cdk5 activity is associated with Niemann-Pick type C (NPC), a neurodegenerative storage disease resulting in premature death in humans.

Regarding claim 31, the Examiner states that it is highly unpredictable as to whether one can use the claimed method for quantitative measure of Cdk5 activity. Applicants have canceled claim 31, making this rejection moot.

The amount of experimentation required is routine

Regarding Claims 23 and 25, the Examiner asserts that it is not routine to determine which, if any, neurological disorders can be detected by an increase in Cdk5 activity, and if so, what level of activity is sufficient to indicate a neurological disorder. Applicants respectfully disagree.

As shown above, an increase in Cdk5 activity is associated with multiple neurological disorders. Knowing whether a sample has increased Cdk5 activity provides useful information which can be taken into account when diagnosing a subject or considering treatment options.

Regarding claim 31, the Examiner asserts that in view of the lack of even a single working example, one of skill must first determine whether the claimed method can be

used for quantitative measure of Cdk5 activity, and if so, establish the details required to quantitatively measure Cdk5 activity.

In an effort to further prosecution, claim 31 has been canceled, rendering this rejection moot.

In view of the above arguments and amendments, all grounds for the rejection under 35 U.S.C. § 112, first paragraph have been obviated or overcome. Reconsideration and withdrawal of these rejections are respectfully requested.

It is believed that all the rejections have been obviated or overcome and the claims are in condition for allowance.

It is not believed that extensions of time or fees for net addition of claims are required. However, in the event that additional extensions of time are necessary to allow consideration of this paper, such extensions are hereby petitioned under 37 CFR § 1.136(a), and any fee required therefore (including fees for net addition of claims) is hereby authorized to be charged to Deposit Account No. 501968.

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CYCLIN-DEPENDENT PROTEIN KINASE 5 ACTIVITY INCREASES IN RAT BRAIN FOLLOWING ISCHEMIA

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Abstract—Cyclin-dependent kinase 5 (CDK5) is the 34 kDa catalytic subunit of a recently characterized neuronal cdc2-like protein kinase which appears to be involved in regulation of the neurocytoskeleton. Using the rat postdecapitative model, the effect of brain ischemia on histone H1 and tau protein CDK5 phosphorylating activity was examined. Histone H1 kinase activity increased in both cytosolic and particulate fractions of the hippocampus and neocortex after 5 min and 15 min of ischemia, then declined to control levels. CDK5 tau protein phosphorylating activity increased after 15 min ischemia; however, no electrophoretic shifts or changes in radiodensity of the tau bands were observed autoradiographically. On Western blot analysis, the CDK5 protein band did not change after 25 min ischemia, despite the increase and subsequent decline in enzyme activity. These data demonstrate a postischemic increase in CDK5 activity, an associated increase in CDK5 tau phosphorylating activity and a decline in activity in the absence of massive proteolysis. CDK5 appears to play a role in the events associated with neuronal response to ischemic injury. © 1997 Elsevier Science Ltd

Cyclin-dependent protein kinase 5 is the catalytic subunit of a recently described neuronal cdc2-like protein kinase (NCLK) (Meyerson *et al.*, 1992; Hellmich *et al.*, 1992; Lew *et al.*, 1992aLew *et al.*, 1992b; Lew and Wang, 1995). Cyclin-dependent kinase 5 (CDK5), also called tau protein kinase II (Kobayashi *et al.*, 1993), brain-derived proline-directed protein kinase (Lew *et al.*, 1992a) or PSSALRE (Meyerson *et al.*, 1992), is a member of a family of Ser/Thr and proline-directed protein kinases (PDPKs) which are related to the cyclin-dependent cell cycle regulatory kinases. CDK5 shares a high sequence homology with $p34^{cdc2}$ (Lew *et al.*, 1992b), displays an identical phosphorylation site specificity, A-A-S/T-P-A-K-A-A-A (Moreno and Nurse, 1990; Beaudette *et al.*, 1993), and may be regulated by a network of protein kinases/phosphatases, inhibitor proteins and cyclin-like proteins which are similar to those which regulate $p34^{cdc2}$.

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Abbreviations: CDK5, cyclin-dependent kinase 5; PDPK, proline-directed protein kinase; MAP/ERK, microtubule-associated protein/extracellular signal-regulated protein kinase.

CDK5 is protein is found at the highest levels in the brain, which is the only tissue that shows significant CDK5 histone H1 kinase activity (Tsai *et al.*, 1994). Immunocytochemical studies have shown that CDK5 is present only in postmitotic neurons (Tsai *et al.*, 1993) but not in glial cells or mitotically active cells. The exact function of CDK5 is unknown, but its counterpart in dividing cells, the $p34^{cdc2}$/cyclin B complex, phosphorylates cytoskeletal components and triggers a G2-M phase transition. CDK5, however, appears to be involved in processes other than cell cycle control. It may be adapted to regulate neurocytoskeletal dynamics, such as neurite outgrowth and cytoskeletal trafficking. *In vitro* neuronal substrates of CDKs include histone H1 (Tsai *et al.*, 1993), synapsin (Hall *et al.*, 1990), neurofilament proteins (Hisanaga *et al.*, 1991; Lew *et al.*, 1992b; Sun *et al.*, 1996; Shetty *et al.*, 1993; Guidato *et al.*, 1996) and tau protein (Manwal-Dewan *et al.*, 1992; Vulliet *et al.*, 1992; Scott *et al.*, 1993; Baumann *et al.*, 1993).

Ischemic brain injury has profound effects on the signal transduction cascades that regulate protein kinase/phosphatase systems (for review see Saitoh *et al.*, 1991). Postischemic alterations in the phosphorylation of microtubule-associated proteins (MAPs) I and II, (Yanagihara *et al.*, 1990; Miyazawa *et al.*, 1993), tau protein (Dewar and Dawson, 1995)

and postischemic activation of MAP kinase, a major PDPK in the brain, have been recently reported (Campos-Gonzalez and Kindy, 1992; Hu and Wieloch, 1994). We propose that another PDPK, CDK5, is also involved in neuronal response to injury and hypothesize that CDK5 activity is altered following "irreversible" brain ischemia. This hypothesis was tested using the postdecapitative rat model for complete ischemia, Western blot analysis and protein kinase assays with immunoprecipitated CDK5 material from the neocortex and hippocampus.

EXPERIMENTAL PROCEDURES

Experimental animals

Postdecapitative global ischemia (Seisjo, 1978) was produced using Wistar rats (275 g). The experimental protocol was approved by the institutional animal care committee. Postdecapitation, the heads were sealed in plastic bags and incubated in a 37°C water bath for 5, 15 or 25 min (six animals per group). The brains were quickly removed and rinsed in ice-cold saline and the meninges were removed. The neocortex and whole hippocampus were then dissected, frozen in liquid nitrogen and stored at −80°C until processed for biochemical analysis. Regions dissected from brains removed in < 60 s served as controls.

Preparation of brain homogenate and enrichment of PDPK activity

Brain homogenates were prepared as crude cytosolic and postmitochrondrial particulate fractions using methods described in Hall *et al.* (1991). In brief, tissues were homogenized in ice-cold buffer (5% w/v) and centrifuged at 1000 *g* for 10 min at 4°C. The supernatant and resuspended particulate fractions were centrifuged for 47 000 *g* for 20 min at 4°C. To enrich for PDPK activity, the fractions were processed by anion-exchange Q-Sepharose FF (Fast Q; Pharmacia) chromatography according to the methods of Vulliet *et al.* (1992) and concentrated in Centricon 10 microfiltration devices (Amicon, Beverly, MA, U.S.A.).

Immunoblotting

Sixty micrograms of protein from the cortex and 30 μg from the hippocampus were loaded into the wells of 10% sodium dodecyl sulfate (SDS)–polyacrylamide gels, electrophoresed and transferred on to nitrocellulose paper (Vulliet *et al.*, 1992). All samples were loaded in series. CDK5 enriched from NIH 3T3 cells (20 μl total cellular lysate; Tsai *et al.*, 1993) was used as a positive control and to confirm the position of rat CDK5. The blots were probed overnight at 4° with an affinity-purified anti-CDK5-CT antibody (1 μg/ml; Upstate Biological, Lake Placid, NY, U.S.A.), incubated for 1 h with alkaline phosphatase-conjugated secondary antibody and developed with an alkaline phosphatase nitroblue dye system. To test for specificity of the anti-CDK5 antibody, a series of samples was immunoblotted with antibodies against other major PDPKs in the brain: CDK4 and MAP/extracellular signal-regulated protein kinase (ERK) (Upstate Biological) and glycogen synthase kinase-3 (GSK-3; Signal Transduction Laboratories, Lexington, KY, U.S.A.). Immunoblots were analyzed for differences in band thickness and intensity by densitometry.

Immunoprecipitation and protein kinase assay

Immunoprecipitation was performed using the same buffers and methods adapted from Hall *et al.* (1991). In brief, concentrated protein (0.50 mg in 200 μl ice-cold immunobuffer) was applied to protein A-Sepharose beads and incubated for 2 h at 4°C with 2.0 μl of the same anti-CDK5-CT antibody described above. Aliquots of both the pelleted immunoprecipitated material and supernatant were assayed for H1 phosphorylating activity. To ensure immunoprecipitation of CDK5 protein from the crude homogenates, Western blots were performed on the pelleted immunoprecipitated material and the supernatant from two complete animal series.

Protein kinase assay

Protein kinase assays were performed using methods adapted from Vulliet *et al.* (1989). Based on the consensus sequence for histone H1, the synthetic peptide analog A-K-A-K-K-T-P-K-K-A-K (UC–Davis Protein Structure Laboratory), a known CDK5 protein kinase substrate (Beaudette *et al.*, 1993), was used to determine activity. As a control substrate, the proline at position 7 was substituted with a glycine (A-K-A-K-K-T-G-K-K-A-K). Additional method controls included the use of non-immunoprecipitated enriched crude homogenate in the reaction mixture, omission of the peptide substrate or boiling brain fractions prior to addition to the reaction mixture. Kinase activity was assayed in the presence of [^{32}P]ATP and 100 μM peptide substrates in a reaction mixture which was incubated for 30 min at 30°C. The supernatants were spotted on to phosphocellulose squares and the amount of ^{32}P incorporated was determined by Cerenkov counting. All assays were performed in triplicate.

CDK5 tau phosphorylating activity was determined using tau purified from the cell extracts of *Escherichia coli* BL2 (DE3) Lys cells transformed with pET-3d plasmid harboring the human four-repeat tau cDNA (Vulliet *et al.*, 1992). Tau (20 μl; 1.40 μg/ml) was added to 45 μl of reaction buffer containing immunoprecipitated CDK5 material from the cytosolic fractions of the neocortex. Kinase activity was assayed in the presence of [^{32}P]ATP in a reaction mixture which was incubated for 1 h at 30°C. The phosphotransferase reaction was terminated by addition of 6 μl of SDS sample buffer and boiling for 5 min. The reaction mixture was resolved on a 10% SDS–polyacrylamide gel. Autoradiography was performed. The bands of interest were cut from the dried gels and the amount of phosphate incorporated was determined by Cerenkov counting.

Statistical analysis for differences in activity between the < 60 s and 5 min, 15 min or 25 min ischemic groups was performed using Dunnett's test. $P < 0.05$ was considered significant.

RESULTS

Complete brain ischemia resulted in a significant increase in histone H1 peptide phosphorylating CDK5 activity after 5 and 15 min in homogenates

from whole hippocampus and neocortex, followed by a decline below control levels after 25 min (Fig. 1). There was an approximate 3-fold and 2-fold increase in activity in the neocortical cytosolic and particulate fractions after 5 min and 15 min ischemia, respectively, although the activity declined markedly after 25 min. Activity increased approximately 1.5-fold in the hippocampal fractions at 5 and 15 min, also followed by a marked decline after 25 min.

On Western blot analysis (Fig. 2), CDK5 appeared as a single 34 kDa band in both the hippocampal and neocortical fractions. There was no band shift or alteration in immunoreactive intensity after 25 min ischemia. A thin non-specific lower molecular mass

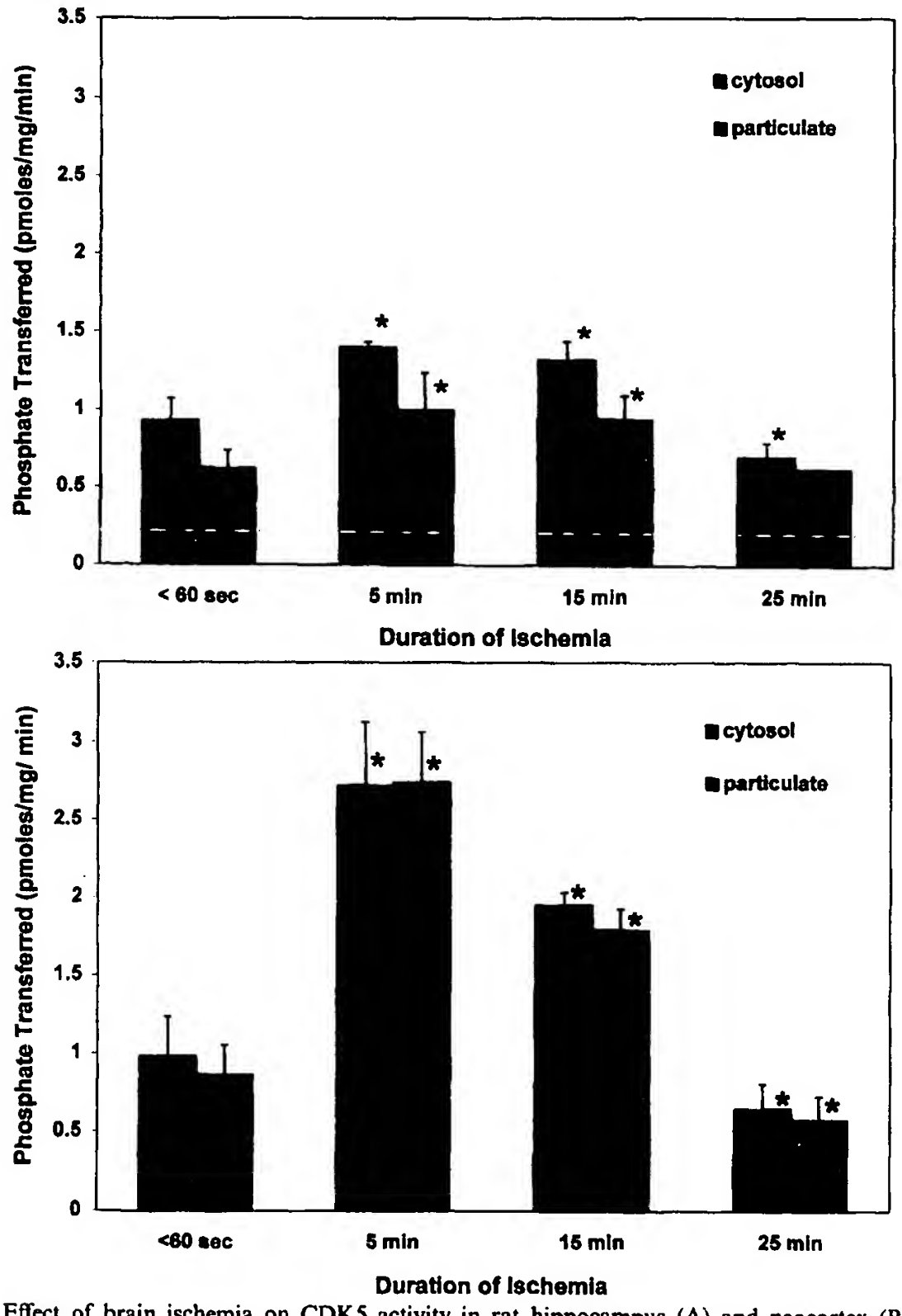

Fig. 1. Effect of brain ischemia on CDK5 activity in rat hippocampus (A) and neocortex (B). Post-decapitation, rat brains were incubated *in situ* at 37°C for 5, 15 or 25 min ($n = 5$ per group). Brains removed in < 60 s served as controls. Cyclin-dependent protein kinase 5 activity was determined in the cytosolic and particulate fractions from neocortex and whole hippocampus by protein kinase assay using immunoprecipitated material. Data are means; error bars indicate the S.D. $^*P < 0.05$ vs < 60 s ischemia. After 5 min and 15 min ischemia, CDK5 activity increased in both regions of the brain, then declined after 25 min.

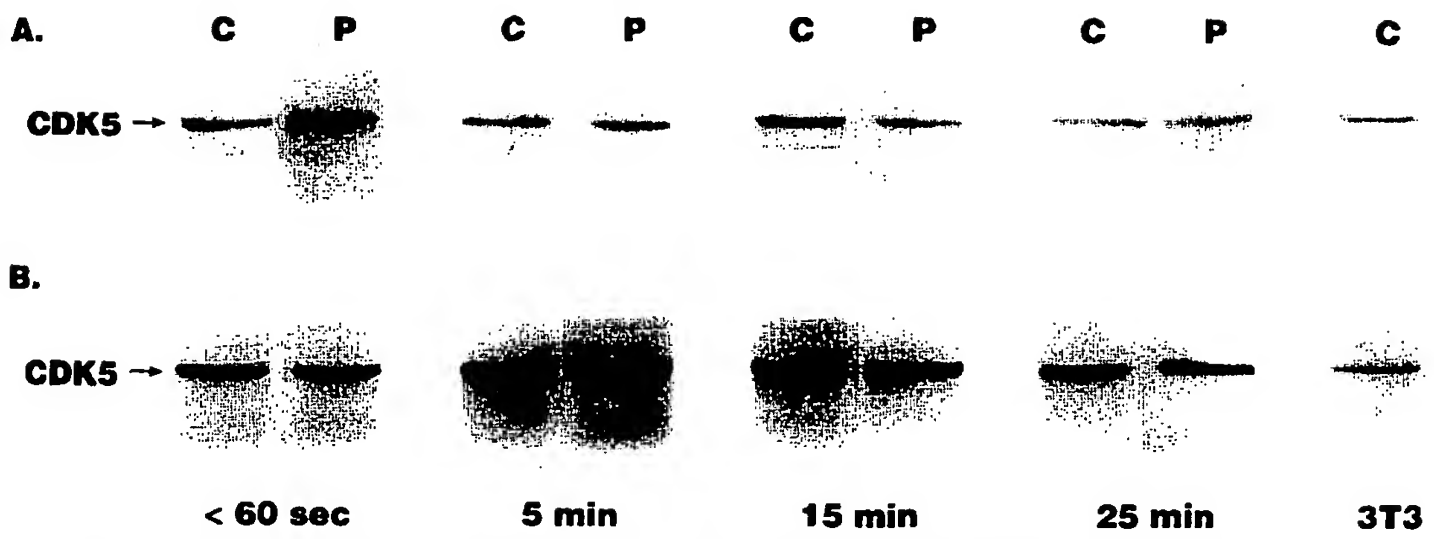

Fig. 2. Immunoblot analysis of CDK5 protein in the cytosolic (C) and particulate (P) fraction of hippocampus (row A) or cortex (row B) after <60 s, 5 min, 15 min or 25 min ischemia. The last lane to the right is CDK5 protein enriched from NIH 3T3 cells. CDK5 appears as a single 34 kDa molecular mass band (arrow). CDK5 levels did not change in either region of the brain after 25 min ischemia.

band inconsistently appeared in some hippocampal fractions after 15 and 25 min ischemia, but evidence of massive proteolysis was not otherwise present. There was no CDK5 protein in the supernatant after immunoprecipitation and no cross-reactivity between the anti-CDK5 antibody and the proteins recognized by the anti-CDK4, MAP/ERK or GSK-3 antibodies (data not shown).

After 15 min ischemia, there was a significant increase (approximately 2-fold) in tau phosphorylation by CDK5 immunoprecipitated material from the neocortical cytosolic fractions (Fig. 3). There was no associated shift in electrophoretic mobility or alteration in the radiodensity of the tau band on autoradiography (data not shown).

DISCUSSION

Our data document alterations in CDK5 activity in the rat brain following irreversible brain ischemia. The postdecapitative rat model of complete ischemia has been extensively used for neurochemical studies and represents a type of brain injury that results from complete cessation of blood flow, prolonged deep energy failure, and subsequent acute and total tissue necrosis (Seisjo, 1978; Lowery *et al.*, 1964; Lowery and Passonneau, 1964). The biochemical and morphological features of this animal model broadly correlate with the type of ischemic brain injury that occurs under certain clinical conditions: cardiac arrest, sepsis, brain edema due to massive head

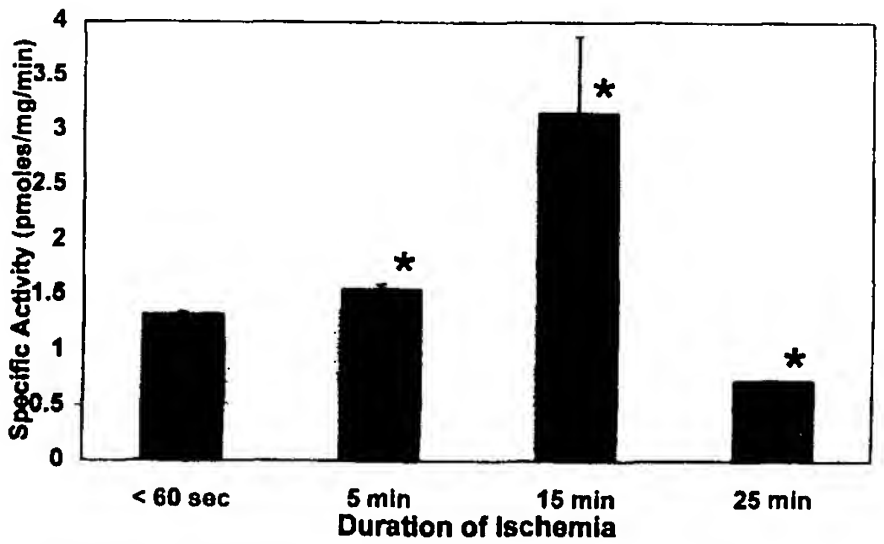

Fig. 3. Postischemic phosphorylation of tau protein by CDK5. Postdecapitation, rat brains were incubated *in situ* at 37°C for 5, 15 or 25 min ($n = 3$ per group). Brains removed in <60 s served as controls. CDK5 tau phosphorylating activity was determined in the cytosolic fractions from neocortex by protein kinase assay using immunoprecipitated material. Data are means; error bars indicate the S.D. $^*P < 0.05$ vs <60 s ischemia. After 15 min ischemia, CDK5 tau phosphorylating activity increased, then declined after 25 min.

trauma, cardioplegia as an adjunctive technique for neurosurgery, or ischemic injury due to severe blood loss. The metabolic responses at the onset of blood flow interruption do not differ qualitatively from those observed after transient global ischemia (Seisjo, 1978). This is also true with regards to the effect of ischemia on many of the components that regulate protein kinase systems in *in vitro* experiments, such as diacylglycerol (Banschbach and Geison, 1974), polyunsaturated fatty acids (Chan *et al.*, 1985; Domanska-Janik *et al.*, 1985) and intracellular Ca^{2+} (Simon *et al.*, 1984; Seisjo and Bengtsson, 1989). Rodent decapitation as a model for irreversible ischemia guarantees that cessation of blood flow is uniformly complete and that neuronal necrosis will occur. Thus decapitation has been a useful method for examining the biochemical events that occur within seconds to minutes after interruption of blood flow to the brain.

The present data were derived from partially purified immunoprecipitated CDK5 material. This supports our proposal that the increase in histone H1 and tau protein phosphorylating activity in these experiments is due specifically to a postischemic increase in CDK5 activity. Brain is the only tissue that shows CDK5 histone H1 kinase activity (Tsai *et al.*, 1994). Furthermore, CDK5 activity in the brain homogenates comes primarily from the neuronal cell population, since CDK5 is not present in either cells of glial origin or neuronal precursor cells (Tsai *et al.*, 1993).

The exact biochemical mechanisms and pathways that regulate CDK5 in the brain have not been extensively characterized. However, activation of its counterpart in dividing cells, $p34^{cdc2}$, requires the presence of cyclin (Solomon *et al.*, 1990), phosphorylation of threonine 161 by cyclin activating kinase, CAK/MO15 kinase (Solomon *et al.*, 1993; Poon *et al.*, 1993; Fequest and Labbe, 1993) and dephosphorylation of Tyr-15 and Thr-14 by cdc25 phosphatase (Parker and Piwnica-Worms, 1992; McGowen and Russell, 1993). Two of the regulatory phosphorylation sites in cdc2, Thr-14 and Tyr-15, and the surrounding amino acid sequences are conserved in CDK5 (Lew and Wang, 1995). In addition, cyclin proteins which activate CDK5 have not been found in the brain, but several neuron-specific CDK5 activator proteins (Lew *et al.*, 1994; Ishiguro *et al.*, 1994; Tsai *et al.*, 1994; Shetty *et al.*, 1995) have been identified.

The lack of change of the CDK5 band on Western blot, despite the time-dependent increase and decline in CDK5 activity, suggests that the kinase is regulated in the postischemic environment by several potential mechanisms: association/disassociation with a regulatory subunit or activator protein, accumulation of the protein as occurs in some cells with DNA damage (Chen *et al.*, 1994; Kastan *et al.*, 1991; Kuerbitz *et al.*, 1992), and/or activation of the existent enzyme via pathways upstream in the signal transduction cascade. The presence of CDK5 protein on immunoblot after 25 min, despite the decline in enzyme activity, suggests inactivation of the protein. The decrease in activity could possibly involve several mechanisms: disassociation of CDK5 from the regulatory subunit or activator protein; deregulation of CDK5 from the regulatory subunit or activator protein; deregulation of protein kinases which regulate CDK5; inhibition of protein synthesis; diminishing ATP reserves and an increase in intracellular Ca^{2+} (conditions that favor phophatases, porteases and dephosphorylation reactions); or the activation of inhibitor proteins. The greater increase in the amount of CDK5 activity in the neocortex, compared with the hippocampus, may reflect the regional variations in the total amount of CDK5 protein and/or regulatory proteins, or selective vulnerability of the hippocampus to ischemia and a greater general depression of signal transduction pathways.

Our data suggest that the postischemic increase in CDK5 activity is also directed towards another known substrate, tau protein. Although the moderate increase in tau phosphorylating activity was significant after 15 min ischemia, no autoradiographic shift in electrophoretic mobility or increased radiodensity of the tau bands was observed. These data are consistent with those described by Paudel *et al.* (1993). CDK5 adds approximately P_i 4 into tau (Baumann *et al.*, 1993); however, visualization of a mobility shift in tau protein probably requires simultaneous phosphorylation of several sites on the molecule.

In Alzheimer's disease, loss of regulatory control of protein kinases/phosphatases, including CDK5, has been proposed to result in altered phosphorylation of tau and in the formation of paired helical filament tau and neurofibrillary tangle pathology (Roush, 1995; Paudel *et al.*, 1993; Baumann *et al.*, 1993; Lui *et al.*, 1995). Paired-helical filament tau and neurofibrillary tangles are not features of ischemic brain injury. However, our data suggest that loss of regulatory control of CDK5 activity occurs after irreversible ischemia in an animal model for stroke.

REFERENCES

Banschbach, M. W. and Geison, R. L. (1974) Postmortem increase in rat cerebral hemisphere diglyceride pool size. *J. Neurochem.* **23**, 875–877.

Baumann, K., Buchan, A. M. and Hill, I. E. (1993) Global ischemia can cause DNA fragmentation indicative of apotosis in rat brain. *Neurosci. Lett.* **164,** 89–92.

Beaudette, K. N., Lew, J. and Wang, J. H. (1993) Substrate specificity characterization of cdc2-like protein kinase purified from bovine brain. *J. Biol. Chem.* **268,** 20825–208230.

Campos-Gonzalez, R. and Kindy, M. R. (1992) Tyrosine phosphorylation of microtubule-associated protein kinase after transient ischemia in the gerbil brain. *J. Neurochem.* **59,** 1955–1958.

Chan, P. H., Fishman, R. A. and Longar, S. (1985) Cellular and molecular effects of polyunsaturated fatty acids in brain ischemia and injury. *Prog. Brain Res.* **63,** 227–235.

Chen, C.-Y., Oliner, J. D. and Zhan, Q. (1994) Interactions between p53 and MDM2 in a mammalian cell cycle checkpoint pathway. *Proc. Natn. Acad. Sci. U.S.A.* **91,** 2684–2688.

Dewar, D. and Dawson, D. (1995) Tau protein is altered by focal cerebral ischemia in the rat: an immunohistochemical and immunoblotting study. *Brain Res.* **684,** 70–78.

Domanska-Janik, K., Lazarewicz, L. and Noremberg, L. (1985) Metabolic disturbances of synaptosomes isolated from ischemic gerbil brain. *Neurochem. Res.* **10,** 573–589.

Fequest, D., Labbe, J.-C. and Devancourt, J. (1993) The MO15 gene encodes the catalytic subunit of a protein kinase that activates cdc2 and other cyclin-dependent kinases (CDKs) through phosphorylation of Thr 161 and its homologues. *EMBO J.* **12,** 3111–3121.

Guidato, S., Tsai, L. H. and Woodgett, J. (1996) Differential cellular phosphorylation and neurofilament heavy sidearms by glycogen synthase kinase-3 and cyclin-dependent kinase-5. *J. Neurochem.* **66**(4), 1698–1706.

Hall, F. L., Braun, R. K. and Mihara, K. (1991) Characterization of the cytoplasmic proline-directed protein kinase in proliferative cells and tissues as a heterodimer comprised of $p34^{cdc2}$ and $p58^{cyclin\ A}$. *J. Biol. Chem.* **266,** 17430–17440.

Hall, F. L., Mitchell, J. P. and Vulliet, P. R. (1990) Phosphorylation of synapsin I at a novel site by proline-directed protein kinase. *J. Biol. Chem.* **265,** 6944–6948.

Hellmich, M. R., Pant, H. C. and Wada, E. (1992) Neuronal cdc2-like protein kinase with predominantly neuronal expression. *Proc. Natn. Acad. Sci. U.S.A.* **89,** 10867–10871.

Hisanaga, S., Kusubata, M. and Okumura, E. (1991) Phosphorylation of neurofilament H subunit at the tail domain by cdc2 kinase disassociate the association to microtubules. *J. Biol. Chem.* **266,** 21798–21803.

Hu, B. and Wieloch, T. (1994) Tyrosine phosphorylation and activation of MAP/ERK protein kinase in the rat brain following transient cerebral ischemia. *J. Neurochem.* **62,** 1357–1367.

Ishiguro, K., Kobayashi, S. and Omori, A. (1994) Identification of the 23 kDa subunit tau protein kinase II as a putative activator of cdk5 in bovine brain. *FEBS Lett.* **324,** 203–208.

Kastan, M. B., Onyekwere, O. and Sidransky, D. (1991) Participation of p53 protein in the cellular response to DNA damage. *Cancer Res.* **51,** 6304–6311.

Kobayashi, S., Ishiguro, K. and Omori, A. (1993) A cdc2-related kinase PSSALRE/cdk5 is homologous with the 30 kDa subunit of tau protein kinase II, a proline-directed protein kinase associated with microtubules. *FEBS Lett.* **335,** 171–175.

Kuerbitz, S. J., Plunkett, B. S. and Walsh, M. B. (1992) Wild-type p53 is a cell cycle checkpoint determinant following irradiation. *Proc. Natn. Acad. Sci. U.S.A.* **89,** 7491–7495.

Lew, J., Beaudette, K. and Litwin, C. M. (1992) Purification and characterization of a novel proline-directed protein kinase from bovine brain. *J. Biol. Chem.* **267,** 13383–13390.

Lew, J., Huang, Q. and Qi, Z. (1994) A brain specific activator of cyclin-dependent kinase 5. *Nature* **371,** 423–426.

Lew, J. and Wang, J. H. (1995) Neuronal cdc2-like kinase. *Trends Biochem. Sci.* **20,** 33–37.

Lew, J., Winkfein, R. J. and Paudel, H. K. (1992) Brain protein kinase is a neurofilament kinase that displays high sequence homology to $p34^{cdc2}$. *J. Biol. Chem.* **267,** 25922–25926.

Lowery, O. H. and Passonneau, J. V. (1964) The relationship between substrates and enzymes of glycolysis in the brain. *J. Biol. Chem.* **239,** 31–42.

Lowery, O. H., Passonneau, J. V. and Hasselberger, F. X. (1964) Effect of ischemia on known substrates and cofactors of the glycolytic pathway in brain. *J. Biol. Chem.* **239,** 18–30.

Lui, W. U., Williams, R. T. and Hall, F. L. (1995) Detection of a cdc2-related kinase associated with Alzheimer paired helical filaments. *Am. J. Path.* **146,** 228–238.

Manwal-Dewan, M., Sen, P. C. and Abel-Ghany, M. (1992) Phosphorylation of tau protein by purified $P34^{cdc28}$ and a related protein kinase from neurofilament. *J. Biol. Chem.* **267,** 10709–19705.

McGowen, G. H. and Russell, P. (1993) Human Wee 1 kinase inhibits cell division by phosphorylating $P34^{cdc2}$ exclusively on Tyr 15. *EMBO J.* **12,** 75–85.

Meyerson, M., Enders, G. H. and Wu, C. L. (1992) A family of cyclin protein kinases. *EMBO J.* **11,** 2909–2917.

Miyazawa, T., Bonnekoh, P. and Hossmann, K. A. (1993) Temperature effect on immunostaining of microtubule-associated protein 2 and synaptophysin after 30 minutes of forebrain ischemia in the rat. *Acta Neuropath.* **85,** 526–532.

Moreno, S. and Nurse, P. (1990) Substrates for $P34^{cdc2}$: *in vivo* veritas. *Cell* **61,** 549–551.

Parker, L. L. and Piwnica-Worms, H. (1992) Inactivation of the $P35^{cdc2}$–cyclin B complex by the human Wee 1 tyrosine kinase. *Science* **257,** 1955–1957.

Paudel, H. K. and Lew, J. and Ali, Z. (1993) Brain proline-directed protein kinase phosphorylates tau on sites that are abnormally phosphorylated in tau associated with Alzheimer's paired helical filaments. *J. Biol. Chem.* **268,** 23512–23518.

Poon, R. Y. C., Yamashita, K. and Adamczewski, J. P. (1993) The cdc2-related protein $P40^{MO15}$ is the catalytic subunit of a protein kinase that can activate $P33^{cdk2}$ and $P34^{cdc2}$. *EMBO J.* **12,** 3123–3132.

Roush, W. (1995) Protein studies try to puzzle out Alzheimer's tangels. *Science* **267,** 793–794.

Saitoh, T., Masliah, E. and Jin, L.-W. (1991) Protein kinases and phosphorylation in neurologic disorders and cell death. *Lab. Invest.* **64,** 596–616.

Scott, C. W., Vulliet, P. R. and Caputo, C. B. (1993) Phosphorylation of Tau by proline-directed protein kinase (p34cdc2/p58cyclinA) decreases Tau-induced microtubule assembly and antibody SM133 reactivity. *Brain Res.* **611,** 237–242.

Seisjo, B. K. (1978) *Brain Energy Metabolism*, p. 454. Wiley Interscience, New York.

Seisjo, B. K. and Bengtsson, F. (1989) Calcium fluxes calcium

antagonists and calcium-related pathology in brain ischemia, hypoglycemia and spreading depression. A unifying hypothesis. *J. Cerebr. Blood Flow Metab.* **4**, 350–361.

Shetty, K. T., Kaech, S. and Link, W. T. (1995) Molecular characterization of a neuronal-specific protein that stimulates the activity of Cdk5. *J. Neurochem.* **64**, 1985–1988.

Shetty, K. T., Link, W. T. and Pant, H. C. (1993) cdc2-like protein kinase from rat spinal cord specifically phosphorylates KSPXX motifs in neurofilament proteins: isolation and characterization. *Proc. Natn. Acad. Sci. U.S.A.* **90**, 6844–6848.

Simon, R. P., Griffiths, T. and Evans, M. C. (1984) Calcium overload in selectively vulnerable neurons in the hippocampus during and after ischemia: an electron microscopy study in the rat. *J. Cerebr. Blood Flow Metab.* **4**, 350–361.

Solomon, M. J., Glotzer, M. and Lee, T. H. (1990) Cyclin activation of p34cdc2. *Cell* **63**, 1013–1024.

Solomon, M., Harper, J. W. and Shuttleworth, J. (1993) CAK the $P34^{cdc2}$ activating kinase contains a protein identical or closely related to $P40^{MO15}$. *EMBO J.* **12**, 3133–3142.

Sun, D., Leung, C. L. and Liem, R. K. (1996) Phosphorylation of the high molecular weight neurofilament protein (NF-H) by CDK5 and p35. *J. Biol. Chem.* **271**, 14245–14252.

Tsai, L., Dellalle, I., Caviness, V. S. Jr (1994) P35 is a neural-specific regulatory subunit of cyclin-dependent kinase 5. *Nature* **371**, 419–423.

Tsai, T. H., Takahashi, T. and Caviness, V. S. (1993) Activity and expression pattern of cyclin-dependent kinase 5 in the embryonic mouse nervous system. *Development* **119**, 1029–1040.

Vulliet, P. R., Hall, F. L. and Mitchell, J. P. (1989) Identification of a novel proline-directed serine/threonine protein kinase in rat pheochromocytoma. *J. Biol. Chem.* **264**, 16292–16298.

Vulliet, R., Halloran, S. M. and Braun, R. K. (1992) Proline-directed phosphorylation of human tau protein. *J. Biol. Chem.* **267**, 22570–22574.

Yanagihara, T., Brengman, J. M. and Mushynski, W. E. (1990) Differential vulnerability of microtubule components in cerebral ischemia. *Acta Neuropath.* **80**, 499–505.

Increase of cdk5 is related to neurofibrillary pathology in progressive supranuclear palsy

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Abstract—*Background:* Progressive supranuclear palsy (PSP) is characterized by a pure neurofibrillary tau pathology involving mainly basal ganglia and brainstem nuclei. In addition to a haplotype of the tau gene potentially favoring tau aggregation, lipoperoxidation has been shown to be associated with PSP tau pathology. *Objective:* To analyze cdk5/p35 complex, a kinase that regulates neurite outgrowth, as a potential cellular mechanism underlying tau phosphorylation in brain tissues from PSP and control cases and comparatively in cerebral cortex from subjects with AD. *Methods:* Cdk5/p35 protein levels and distribution were evaluated by immunoblotting and immunocytochemistry in brain regions from seven PSP, six AD, and seven control cases, with similar postmortem intervals. *Results:* Total cdk5 protein levels were significantly increased by more than threefold in PSP tissue and were augmented in PSP neurons, codistributed with tau immunoreactivity. P35, the regulatory subunit of cdk5, was degraded by postmortem proteolysis to the same extent in PSP, AD, and control tissues. *Conclusions:* The proteolysis in vivo of p35, the regulatory subunit of the kinase, is not ascertainable because it is masked by its postmortem degradation. The study, however, indicates that in PSP, the alteration of cdk5 is different from that described in AD and suggests that the absence of amyloid β protein deposition may account for the different pathways responsible for the same kinase activation.

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Progressive supranuclear palsy (PSP) is classified as a sporadic form of tauopathy, a term that includes a heterogeneous group of neurodegenerative disorders characterized by intracellular aggregation of abnormally phosphorylated tau protein in the absence of other distinctive pathologic features.[1,2] Tau protein aggregation, which leads to the formation of abnormal cytoskeletal fibrils, is supposed to be the primary pathogenic event of these pathologies. Indeed, in favor of this hypothesis, familial forms of tauopathies result from various mutations of the tau gene.[3-5] Moreover, sporadic tauopathies such as PSP and corticobasal degeneration are associated with a specific haplotype of the tau gene,[6-8] suggesting that tau aggregation is favored by common polymorphisms within the gene encoding it.

However, since PSP is a late-onset sporadic disease, age-related factors are likely to be implicated in its degenerative process. Oxidative stress is directly correlated to aging and is linked to the pathologic process of PSP. Toxic products of lipid peroxidation, such as 4-hydroxynonenal (HNE) and malondialdehyde, are selectively increased in PSP brain tissue, and their occurrence is proportional to the extent of tau pathology.[9-11] Such lipid peroxidation products promote microtubule disassembly as well as tau hyperphosphorylation in cultured neuronal cells[12,13] and also affect tau conformational change and assembly.[14,15] These events might be determined by an increased activation of cdk5, a kinase physiologically involved in tau phosphorylation. Indeed, an abnormal processing of p35, the regulatory subunit of cdk5, has been described in AD and is indicated as a major cause of tau phosphorylation and aggregation,[16] which together with extracellular deposition of amyloid β protein (Aβ) represent the characteristic lesion of the disease. In AD, oxidative stress is suggested as the trigger of cdk5 activation through calpain-mediated cleavage of p35.[17] Altogether, these findings suggest that such pathogenic events may occur in PSP, in which a strict relationship between lipid peroxidation and tau aggregation exists.

To investigate this issue, we studied the cdk5/p35 system and its relationship with the distribution of tau pathology in brain tissue from PSP cases. In parallel, the same analysis was carried out in cerebral cortex from subjects with AD, in which Aβ deposition is suggested to play a primary role in generating oxidative stress as well as neurofibrillary pathology.

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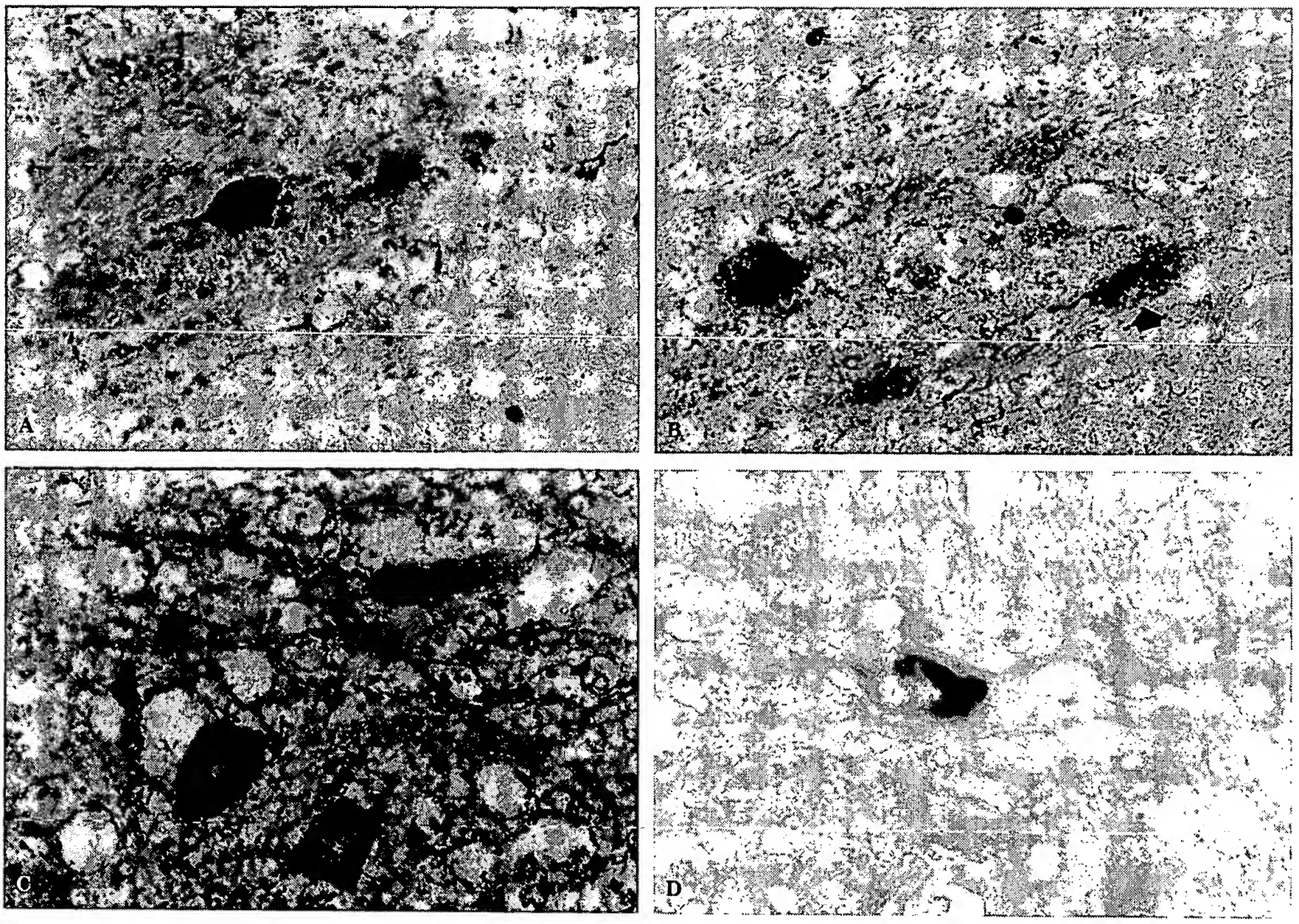

Figure 1. In PSP, cdk5 immunoreactivity (B, arrow) is increased in a pontine neuron that is tau reactive (A). Some non–tangle-bearing pontine neurons display cdk5 immunoreactivity (C). Double immunolabeling shows colocalization of diffuse cdk5 reactivity (brown) with tau staining (red) (D). Original magnification ×600.

Materials and methods. *Tissues.* Seven cases fulfilling the clinical and pathologic National Institute of Neurological and Communication Disorders and Stroke criteria for PSP[18] (63–78 years old), six cases with pathologically confirmed AD (68–83 years old), and seven age-matched control subjects (65–80 years old) were considered. The three groups of cases were matched for the postmortem intervals that varied from 8 to 10 hours. Brains were removed at autopsy, and adjacent blocks of cerebral cortex, basal ganglia, midbrain, pons, and cerebellum were either fixed in 10% formalin or stored unfixed at −80 °C. In each PSP case, we selected for biochemical analysis three brain regions (motor cortex, thalamus, and striatum) with a high degree of tau pathology, ascertained by immunocytochemistry in sections cut from adjacent blocks. In each PSP case, the extent of tau pathology was expressed as the percentage of tau-reactive neurons of the total neuronal number. In AD cases, the same analysis was carried out in blocks of frontal cortex (superior frontal gyrus). Blocks from the same four regions were selected in control cases.

Immunocytochemistry. Immunocytochemistry was performed in formalin-fixed, paraffin-embedded sections of pons in all three groups of cases. Serial sections were processed according to the biotin–avidin method by using monoclonal antibody AT8 (Innogenetics, Temse, B.; Ghent, Belgium) to tau (1:100) and antisera against cdk5 (1:100) and the C terminus of p35 (p25) (1:200).[16] The reaction was developed with 3,3'-diaminobenzidine as co-substrate. For double immunostaining, sections were incubated with antiserum against cdk5 and monoclonal antibody AT8, using the biotin–avidin and the alkaline phosphatase–antialkaline phosphatase methods and, respectively, diaminobenzidine and fast red as chromogens.

Immunoblot analysis. Brain tissues (100 mg; motor cortex, thalamus, and striatum in PSP cases; frontal cortex in AD subjects; all four regions in control subjects) were homogenized in Laemmli's sample buffer 1:10 (wt/vol) containing 10% sodium dodecyl sulfate (SDS) and heat treated. Thirty microliters of brain homogenates was separated by 12.5% SDS polyacrylamide gel electrophoresis and transferred onto polyvinylidene difluoride membranes (90 V for 2 hours). Membranes were blocked with 5% dry milk and incubated at 4 °C for 12 hours with the following primary antibodies: (1) polyclonal antiserum p10 recognizing the N terminus of p35 (1:1,000)[16]; (2) polyclonal antiserum p25 specific for the C terminus of p35 (1:1,000)[16]; and (3) monoclonal antibody DC 17 recognizing cdk5 (1:1,000) (Biotec, Santa Cruz, CA). After incubation with horseradish peroxidase-conjugated secondary antibodies (Amersham, Buckinghamshire, UK) that was performed at room temperature for 1 hour, the reaction was visualized with ECL-Plus (Amersham). The reactivity was quantified

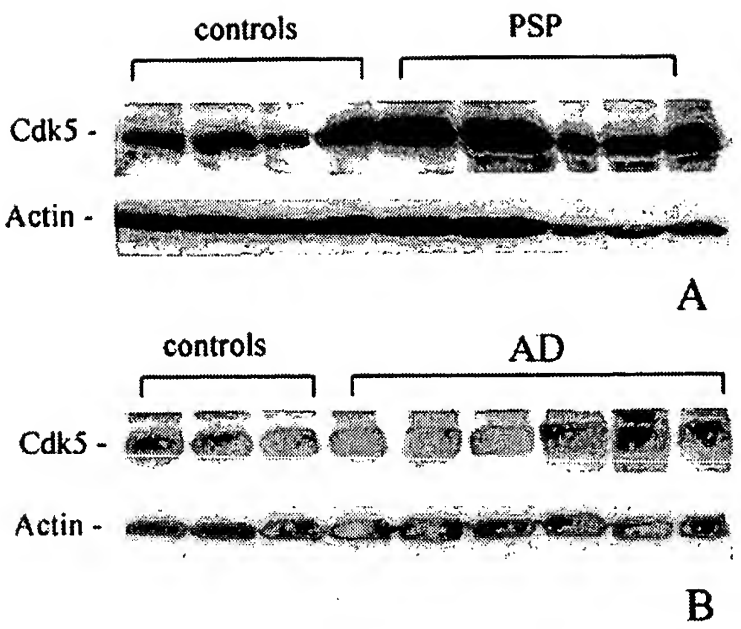

Figure 2. Cdk5 and actin immunoblots of brain lysates from cases of progressive supranuclear palsy (PSP) and control cases (A; motor cortex) and from cases of AD and control cases (B; superior frontal gyrus). Cdk5, migrating at 34 kD, is constantly elevated in PSP cases, whereas no quantitative difference exists between AD and control cases. The lower actin reactivity of some PSP cases (A; last three lanes) reflects the lower amount of total proteins.

using Quantity One software (Bio-Rad, Hercules, CA). To normalize protein levels, membranes were stripped with Immunopure (Pierce, Rockford, IL) and then probed with a monoclonal antibody against actin (1:6,000) (Sigma, St. Louis, MO). The absolute values of ckd5, p35, and p25/p35 were then normalized to the corresponding values of actin and statistically analyzed using a nonparametric test.

Results. *Immunocytochemistry.* The monoclonal antibody (AT8) against tau recognized neurons showing intracellular abnormal fibrils in the tegmentum of pons in PSP (figure 1A) as well as AD cases. In PSP cases, about 10% of tau-reactive neurons displayed also cdk5 immunoreactivity (see figure 1B), and a global but weak increase of cdk5 immunoreactivity was observed in non-tangle-bearing cells (see figure 1C). Double immunolabeling showed colocalization of diffuse cytoplasmic cdk5 reactivity with tau staining in PSP pontine neurons (see figure 1D). However, in AD and in control tissue, cdk5 reactivity was completely absent (not shown). The antibodies against p35 (either N or C terminus specific) also weakly immunolabeled few neurons in PSP and AD cases (without relationship with tau reactivity) and control tissues (not shown).

Immunoblot analysis. Cdk5 was identified with the specific antibody as a single band with the expected molecular mass of 34 kD (figure 2). The antibody p35, specific for the C terminus of the regulatory subunit p35, recognized two peptides migrating at 35 and 25 kD, corresponding to the full-length protein and its C-terminal proteolytic product, p25 (figure 3). The amount of the latter peptide was consistently higher than that of the entire peptide, as demonstrated by the calculated p25/p35 ratios (see above and figure 3). The average protein level of cdk5 in PSP tissues was 3.9-fold that in control tissues (9.1 ± 2.5 in PSP and 2.3 ± 0.8 in controls; arbitrary units; means of values of three regions; $p < 0.001$) (see figure 2A). No statistical difference between AD and control cases was found (2.6 ± 0.4 in AD and 2.7 ± 0.7 in controls) (see figure 2B). Moreover, no statistical differences among the three areas examined (motor cortex, thalamus, nucleus caudatus) were found in PSP or in control cases. In PSP cases, a strong correlation between the extent of tau pathology and cdk5 level was detected (correlation index = 0.975). The average total amount of the regulatory subunit p35 was weakly decreased in PSP and AD cases in comparison with the corresponding control tissues, without statistical difference (1.6 ± 0.2 in PSP and 2.1 ± 1.6 in controls; means of values of three regions; 1.8 ± 0.8 in AD and 1.9 ± 0.7 in controls) (see figure 3). Similarly, the average p25/p35 ratio was similar in PSP, AD, and control tissues (2.7 ± 1.5 in PSP and 2.4 ± 0.87 in controls; 1.9 ± 1.1 in AD and 2.2 ± 0.8 in controls) (see figure 3).

Discussion. The current study demonstrates that cdk5 protein levels are increased by 3.9-fold in affected brain regions in PSP and that cdk5 immunoreactivity is especially augmented in neurons showing tau protein accumulation. Cdk5 is a kinase that physiologically promotes neurite outgrowth through phosphorylation of cytoskeletal molecules, including tau.[19,20] Increased activity of cdk5, mediated by the excessive cleavage of its regulatory subunit p35, has been shown to occur in AD and is indicated as a major mechanism of neurofibrillary tangle formation.[16] Cleavage of p35, leading to the accumulation of its C-terminal fragment, p25, is induced in vivo by calpain, a calcium-dependent protease.[17] However, quick postmortem degradation of p35 produces, within 1 to 6 hours, high levels of p25 such as to mask its actual increase in vivo.[21,22] In-

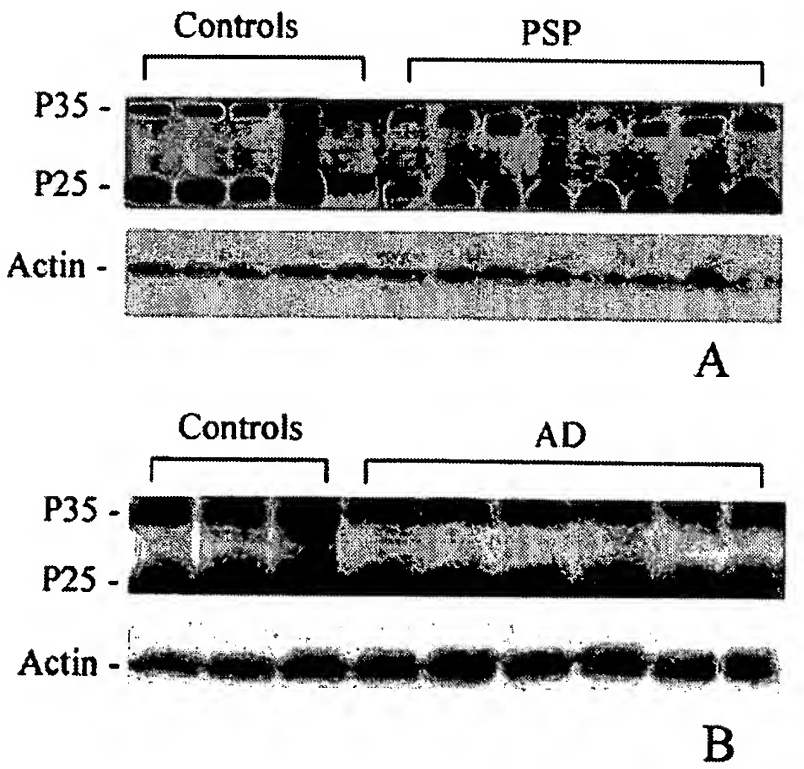

Figure 3. p35 and actin immunoblots of brain lysates from cases of progressive supranuclear palsy (PSP) and control cases (A; motor cortex) and from cases of AD and control cases (B; superior frontal gyrus). p35 and its proteolytic product, p25, are not varied in normal and pathologic tissues. The amount of p25 is greater than holoprotein p35 in all cases.

deed, in agreement with recent reports,[21,22] we observed that the proteolytic fragment p25 was two to three times more abundant than the holoprotein p35 in all cases, without significant differences among pathologic and control subjects. Since the postmortem cleavage of p35 hampers a correct evaluation of its proteolysis in vivo, the possible pathogenic role of the regulatory subunit cannot be ascertained directly in brain tissue affected by either AD and PSP, unless using tissues with very short postmortem delay, under 3 hours.[16]

Differently from the case in AD, protein levels of cdk5, the catalytic subunit of the complex, are significantly increased in PSP brain tissue. The increase of cdk5 is likely to depend on gene overexpression and not on defective clearance. We did not confirm this hypothesis by doing quantitative cdk5 transcript analysis, since we have found that RNA from the majority of PSP cases was degraded, for unknown reasons. Lipoperoxidative products such as HNE and malondialdehyde that specifically accumulate in PSP-affected brain regions are likely to trigger cdk5 overexpression. Indeed, low concentrations of HNE, similar to those detected in PSP tissue, cause in neuronal cells increase of cdk5 protein levels, activity, and transcript, without affecting proteolysis of the regulatory subunit p35 (P. Strocchi et al., manuscript in preparation).

The qualitatively different alteration of the cdk5 system observed in PSP compared with AD is probably related to the absence or presence of Aβ, which drives different pathways leading to neurodegeneration as well as neurofibrillary pathology. In a previous study, we demonstrated a similar distinction for lipoperoxidation damage that is selectively involved in the tau pathology of PSP, whereas in AD, all oxidative markers are elevated.[11]

The current study suggests that cdk5 mediates the noxious effect of lipoperoxidation on tau protein aggregation through the overproduction of the kinase involved in tau protein phosphorylation. Finally, our findings further support the hypothesis of oxidative stress as an extragenetic factor of PSP pathogenesis and suggest the need for trials with antioxidant compounds in patients with PSP.

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References

1. Dickson DW. Neurodegenerative diseases with cytoskeletal pathology: a biochemical classification. Ann Neurol 1997;42: 541–544.
2. Spillantini MG, Goedert M. Tau protein pathology in neurodegenerative diseases. Trends Neurosci 1998;21:428–433.
3. Bugiani O, Murrel JR, Giaccone G, et al. Frontotemporal dementia and corticobasal degeneration in a family with a P301 mutation in tau. J Neuropathol Exp Neurol 1999;58:667–677.
4. Goedert M, Spillantini MG, Crowther RA, et al. Tau gene mutation in familial progressive subcortical gliosis. Nat Med 1999;5:454–457.
5. Spillantini MG, Murrell JR, Goedert M, Farlow MR, Klug A, Ghetti B. Mutation in tau gene in familial multiple system tauopathy with presenile dementia. Proc Natl Acad Sci USA 1998;95:7737–7741.
6. Bennet P, Bonifati V, Bonuccelli U, et al. Direct genetic evidence for involvment of tau in progressive supranuclear palsy. European Study Group on Atypical Parkinsonism Consortium. Neurology 1998;51:982–985.
7. Baker M, Litvan I, Houlden H, et al. Association of an extended haplotype in tau gene with progressive supranuclear palsy. Hum Mol Genet 1999;8:711–715.
8. Di Maria E, Tabaton M, Vigo T, et al. Corticobasal degeneration shares a common genetic background with progressive supranuclear palsy. Ann Neurol 2000;47:374–377.
9. Albers DS, Augood SJ, Martin DM, Standaert DG, Vonsattel JP, Beal MF. Evidence for oxidative stress in the subthalamic nucleus in progressive supranuclear palsy. J Neurochem 1999; 73:881–884.
10. Albers DS, Beal MF. Mitochondrial dysfunction and oxidative stress in aging and neurodegenerative disease. J Neural Transm (Suppl) 2000;59:133–154.
11. Odetti P, Garibaldi S, Norese R, et al. Lipoperoxidation is selectively involved in progressive supranuclear palsy. J Neuropathol Exp Neurol 2000;59:393–397.
12. Neely MD, Sidell KR, Graham DG, Montine TJ. The lipid peroxidation product 4-hydroxynonenal inhibits neurite, disrupts neuronal microtubules, and modifies cellular tubulin. J Neurochem 1999;72:2323–2333.
13. Mattson MP, Fu W, Waeg G, Uchida K. 4-Hydroxynonenal, a product of lipid peroxidation, inhibits dephosphorylation of the microtubule-associated protein tau. Neuroreport 1997;7: 2275–2281.
14. Takeda A, Smith MA, Avila J, et al. In Alzheimer's disease, heme oxygenase is coincident with ALZ50, an epitope of tau induced by 4-hydroxy-2-nonenal modification. J Neurochem 2000;75:1234–1241.
15. Perez M, Caudros R, Smith MA, Perry G, Avila J. Phosphorylated, but not native, tau protein assembles following reaction with the lipid peroxidation product, 4-hydroxy-2-nonenal. FEBS Lett 2000;486:270–274.
16. Patrick GN, Zuckerberg LM, Nikolic, De La Monte S, Dikkes P, Tsai L-H. Conversion of p35 to p25 deregulates Cdk5 activity and promotes neurodegeneration. Nature 1999;402:615–622.
17. Lee MS, Known YT, Li M, Peng J, Friedlander RM, Tsai L-H. Neurotoxicity induces cleavage of p35 to p25 by calpain. Nature 2000;405:360–364.
18. Litvan I, Hauw JJ, Bartko JJ, et al. Validity and reliability of the preliminary NINDS neuropathologic criteria for progressive supranuclear palsy and related disorders. J Neuropathol Exp Neurol 1996;55:97–105.
19. Nikolic M, Dudek H, Known YT, Tsai LH. The Cdk5/p35 kinase is essential for neurite outgrowth during neuronal differentiation. Genes Dev 1996;10:816–825.
20. Paglini G, Pigino G, Kunda P, et al. Evidence for the participation of the neuron-specific Cdk5 activator p35 during laminin-enhanced axonal growth. J Neurosci 1998;18:9858–9869.
21. Taniguchi S, Fujita Y, Hayshi S, et al. Calpain-mediated degradation of p35 to p25 in postmortem human and rat brains. FEBS Lett 2001;489:46–50.
22. Yoo B, Lubec G. P25 protein in neurodegeneration. Nature 2001;411:763–764.

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Deregulation of cdk5, Hyperphosphorylation, and Cytoskeletal Pathology in the Niemann–Pick Type C Murine Model

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NPC-1 gene mutations cause Niemann–Pick type C (NPC), a neurodegenerative storage disease resulting in premature death in humans. Spontaneous mutation of the *NPC-1* gene in mice generates a similar phenotype, usually with death ensuing by 12 weeks of age. Both human and murine NPC are characterized neuropathologically by ballooned neurons distended with lipid storage, axonal spheroid formation, demyelination, and widespread neuronal loss. To elucidate the biochemical mechanism underlying this neuropathology, we have investigated the phosphorylation of neuronal cytoskeletal proteins in the brains of *npc-1* mice. A spectrum of antibodies against phosphorylated epitopes in neurofilaments (NFs) and MAP2 and tau were used in immunohistochemical and immunoblotting analyses of 4- to 12-week-old mice. Multiple sites in NFs, MAP2, and tau were hyperphosphorylated as early as 4 weeks of age and correlated with a significant increase in activity of the cyclin-dependent kinase 5 (cdk5) and accumulation of its more potent activator, p25, a proteolytic fragment of p35. At 5 weeks of age, the development of axonal spheroids was noted in the pons. p25 and cdk5 coaccumulated with hyperphosphorylated cytoskeletal proteins in axon spheroids. These various abnormalities escalated with each additional week of age, spreading to other regions of the brainstem, basal ganglia, cerebellum, and eventually, the cortex. Our data suggest that focal deregulation of cdk5/p25 in axons leads to cytoskeletal abnormalities and eventual neurodegeneration in NPC. The *npc-1* mouse is a valuable *in vivo* model for determining how and when cdk5 becomes deregulated and whether cdk5 inhibitors would be useful in blocking NPC neurodegeneration.

Key words: cdk5; p35; neurodegeneration; Niemann–Pick disease type C; cholesterol; axon spheroid; lipid rafts; caveolas; neurofilament phosphorylation; tau phosphorylation; cytoskeletal pathology

Niemann–Pick type C disease (NPC) is a rare, autosomal recessive, fatal, lysosomal lipidosis affecting multiple organs (Vanier et al., 1991a,b; Scriver et al., 2001). The disease is caused predominantly by mutations in the *NPC-1* gene and less frequently in the *HE1* (also referred to as *NPC-2*) gene (Naureckiene et al., 2000; Millat et al., 2001; Scriver et al., 2001). The *NPC-1* gene encodes for a cholesterol transporter in late endosomes, and the *HE1* gene encodes for a lysosomal cholesterol-binding protein. Neuropathologically, NPC is characterized by neurons distended with lipid storage material having a foamy appearance, dendritic and axonal abnormalities, demyelination, and widespread neuronal loss (Elleder et al., 1985; Love et al., 1995; Suzuki et al., 1995). In addition, neurofibrillary tangles (NFTs), a diagnostic lesion of Alzheimer's disease (AD), are also a consistent finding, particularly in cases with a prolonged course of disease (Auer et al., 1995; Love et al., 1995; Suzuki et al., 1995) (H. H. Klünemann, B. Bu, J. Husseman, M. Elleder, K. Suzuki, S. Salamant, S. Love, H. Budka, C. Fligner, T. Bird, L.-W. Jin, D. Nochlin, and I. Vincent, unpublished observations). How these various neuropathologic features result from altered cholesterol metabolism in NPC is a mystery and a rather difficult one to resolve given the rarity of the disease.

A tremendous asset for unraveling the neuropathologic effects of *NPC-1* mutations is the BALB/cNpc-1nih mouse, which harbors a spontaneous mutation in its *npc-1* gene (Loftus et al., 1997). Mice with homozygous *npc-1* mutations (*npc-1* mice) display extensive lipid storage accumulation, neuroaxonal dystrophy, and neuronal loss, similar to that of human NPC (Higashi et al., 1993; Suzuki et al., 1995; Sawamura et al., 2001). Cholesterol (Xie et al., 1999; Sawamura et al., 2001) and glycosphingolipids such as gangliosides GM2 and neutral glycolipids (Walkley, 1995; Zervas et al., 2001) are the predominant constituents of storage material in the *npc-1* mouse brain. Curiously, however, neither alleviation of cholesterol (Patterson et al., 1993; Erickson et al., 2000; Camargo et al., 2001) nor ganglioside storage (Liu et al., 2000) ameliorate the neurological phenotype or progressive neuronal loss in *npc-1* mice or feline NPC, although lipid storage was effectively reduced in neurons and other cells. Thus, it is yet unclear what mechanism underlies neuronal dysfunction and loss of neurons in NPC. A notable difference between the *npc-1* mouse and human NPC is the absence of NFTs in the mouse (German et al., 2001a; Sawamura et al., 2001). However, in light of the conspicuous axonal abnormalities in human, murine, and feline NPC (Elleder et al., 1985; Higashi et al., 1993; Ong et al., 2001), we wondered whether cytoskeletal abnormalities contribute to neuronal dysfunction and degeneration in NPC. Therefore,

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Table 1. Antibodies and specificities

Antibody types	Specificity	Isotype	Source
Phosphotau			
PHF-1	Ser 396-404	Mouse IgG1	P. Davies
CP-13	Ser 202	Mouse IgG1	P. Davies
CP-10	Thr 231	Mouse IgM	P. Davies
CP-22	Ser 175	Mouse IgM	P. Davies
MC-6	Thr 235	Mouse IgG1	P. Davies
Sequence-tau			
TG-5	220-240	Mouse IgG1	I. Vincent/P. Davies
ALZ-50	5-15, 312-322	Mouse IgM	P. Davies
MC-1	5-15, 312-322	Mouse IgG1	P. Davies
Cytoskeletal			
SMI 31	Phospho-NF-H and -NF-M	Mouse IgG1	Sternberger Monoclonals, Inc. (Lutherville, MD)
SMI 32	Nonphospho-NF-H	Mouse IgG1	Sternberger Monoclonals, Inc.
R39	Total NF	Rabbit	H. C. Pant (National Institutes of Health/National Institute of Neurological Disorders and Stroke, Bethesda, MD)
AP-18	Phospho-MAP2	Mouse IgG1	L. Binder (Northwestern University, Chicago, IL)
AP-20	MAP2a + MAP2b + MAP2c	Mouse IgG1	Sigma
Kinase			
cdk5 (C-8)	cdk5	Rabbit	Santa Cruz Biotechnologies (Santa Cruz, CA)
cdk5 (DC-17)	cdk5	Mouse IgG1	Santa Cruz Biotechnologies
p35 (C-19)	C-terminal p35	Rabbit	Santa Cruz Biotechnologies
p35 (N-20)	N-terminal p35	Rabbit	Santa Cruz Biotechnologies
Phospho-GSK-3β	phosphoSer9 of human GSK-3β	Rabbit	Cell Signaling Technology (Beverly, MA)
GSK-3β	N-end human GSK-3β	Rabbit	Cell Signaling Technology
Loading control			
Anti-NeuN	NeuN	Mouse IgG1	Chemicon International (Temecula, CA)

we have undertaken a detailed characterization of cytoskeletal protein phosphorylation in the brains of *npc-1* mice.

MATERIALS AND METHODS

All procedures in this study were approved by the Internal Review Board and Animal Use and Care Committee of the University of Washington.

Npc-1 mice. A breeding pair of heterozygous *npc-1* mice obtained from The Jackson Laboratory (Bar Harbor, ME) was bred to generate wild-type (+/+), heterozygous (*npc-1* +/−), and homozygous (*npc-1* −/−) mice, which were identified using an established PCR-based method (Loftus et al., 1997). Tail biopsies for genotyping were performed at the time of weaning (i.e., at ~3 weeks). Only −/− mice have been reported to display pathology (Tanaka et al., 1988). In initial studies, we screened +/− mice and confirmed the absence of cytoskeletal pathology in this genotype. Hence, all further study concentrated on comparisons of −/− mice with +/+ siblings. Twenty-eight *npc-1* −/− mice (4, 5, 7, and 9 weeks of age, $n = 3$; 6 and 8 weeks of age, $n = 5$; 10, 11, and 12 weeks of age, $n = 2$) and a minimum of two age-matched (for each week), wild-type littermates were analyzed by immunohistochemistry and immunoblotting.

Brain tissue. Mice were killed by carbon dioxide exposure followed by decapitation. The brains were removed quickly and divided sagittally into halves. The right halves were immersion fixed with 4% paraformaldehyde/PBS for 1 week and then embedded in paraffin. Where indicated, some mice were transcardially perfused with 4% paraformaldehyde/PBS, and the brain was then processed for paraffin embedding. The paraffin-embedded blocks were sectioned at 6 μm for histological analyses. The left halves were frozen at −80°C for biochemical study. In some cases, the forebrain, cerebellum, and brainstem were isolated and frozen separately for regional analysis.

Frozen hippocampus from a clinically and neuropathologically confirmed AD case was used in parallel with the mouse samples as a control for specificity of NFT antibodies.

Antibodies. The primary antibodies used in this study are summarized in Table 1.

Immunohistochemistry and immunofluorescent labeling. Immunohistochemical staining was performed on paraffin-embedded sections as described previously (Vincent et al., 1997, 1998) with modifications. Immersion-fixed sections were incubated with 88% formic acid for 7 min to enhance antigen recovery and washed three times for 15 min with Tris-buffered saline (in mM): 10 Tris-HCl, pH 7.4, and 150 NaCl, before the addition of primary antibody. Biotinylated, isotype-specific secondary antibodies followed by HRP-labeled streptavidin were used to detect primary antibody-specific binding, visualized using diaminobenzidine (DAB, brown). Sections were counterstained with hematoxylin (blue-purple).

Immunofluorescent labeling was conducted similarly (Vincent et al., 1998). For visualization of specific antibody binding, streptavidin-conjugated Cy-3 (red, 1:500) or Alexa Fluor 488 (green, 1:500) was used. Nuclei were counterstained in 1 μg/ml 4,6-diamino-2-phenyliodole (DAPI, blue) in distilled water for 30 sec at room temperature. Light and fluorescent micrographs were collected using a Nikon (Tokyo, Japan) Optiphot microscope connected to a computerized SPOT CCD camera (Diagnostic Instruments, Inc., Sterling Heights, MI).

Preparation of brain extracts. Frozen tissue was weighed and homogenized with a polytron in 10 volumes of ice-cold lysis buffer [in mM: 10 Tris-HCl, 150 NaCl, 20 NaF, 1 mM sodium vanadate, 2 ethylene glycol-bis (β-aminoethylether)-*N,N,N',N'*-tetraacetic acid, 0.5% Triton X-100, and 0.1% SDS] and proteinase inhibitor cocktail (P-8340; Sigma, St. Louis, MO). The homogenates were aliquoted and stored at −80°C.

Immunoblotting analysis. Frozen aliquots were thawed and centrifuged at 12,000 × *g* at 4°C for 5 min, and the soluble fraction was used for immunoblotting. The protein content in supernatants was determined using a Bio-Rad (Hercules, CA) protein assay kit and a Microplate Reader (Molecular Devices, Sunnyvale, CA). The supernatants were then subjected to SDS-gel electrophoresis and immunoblotting analyses

(Vincent et al., 1997). For the analyses of MAP2 and neurofilaments (NFs), 10 μg of protein was loaded per lane; for tau and cyclin-dependent kinase 5 (cdk5) studies, 40 μg of protein was loaded per lane. For heat-stable proteins, supernatants containing 30 μg of protein were boiled for 10 min at 100°C and then centrifuged. The supernatants containing the heat-soluble protein were resolved and immunoblotted according to routine procedures.

Immunoprecipitation. Protein (100 μg) from lysates in equal volume was immunoprecipitated with 2 μg of cdk5 polyclonal antibody as described previously (Vincent et al., 1997). The immunoprecipitates (IPs) were processed for immunoblotting (as above) or kinase assay as described below.

Kinase assay. The cdk5 IPs were washed twice with lysis buffer and once with kinase buffer (50 mM HEPES, pH 7.0, 10 mM $MgCl_2$, 1 mM dithiothreitol, and 10 μM cold ATP), resuspended in 20 μl of kinase buffer containing 10 μg of histone H1 and 0.5 μCi of [γ-^{32}P]ATP, and incubated at room temperature for 20 min. To stop the reaction, 5 μl of 5× sample buffer was added, and the samples were boiled for 5 min at 95°C. Samples were separated by SDS-PAGE. Resolved proteins in the gel were visualized with Coomassie blue dye, and the gels were then dried and exposed to film for autoradiography.

Densitometric analysis. ECL and autoradiographic films were scanned, the appropriate bands were outlined, and their densities were measured using NIH image software. Statistical differences were determined with Student's *t* test using Microsoft Excel (Seattle, WA)

RESULTS

Hyperphosphorylation and accumulation of neurofilament protein in *npc-1* −/− mouse brain

The immunohistochemical staining with SMI 31 antibody was performed on immersion-fixed sagittal sections. Consistent with the expected localization of phosphorylated NFs (Julien and Mushynski, 1982), SMI 31 primarily stained axon tracts throughout the brain of wild-type (+/+) mice (Fig. 1*A,C,E*). In *npc-1* −/− mice, SMI 31 stained numerous spot-like structures in the same regions through which axons typically course (Fig.1*B,D,F* shown at higher magnification in Fig.1*G,H*, *red*, *I*, *arrowhead*). The abnormal spot-like structures were first noted in the pons at 5 weeks of age; they were present throughout the basal ganglia (Fig. 1*B*), brainstem (Fig. 1*D*), and white matter of the cerebellum (data not shown) by 7–8 weeks of age but were rarely seen in the hippocampus (Fig. 1*F*, *arrows*). By 11–12 weeks of age, they were observed in small numbers in the cerebral cortex as well (data not shown). Although we did not directly analyze neuronal loss in this study, disruption of the continuity of the Purkinje neuron layer in the cerebellum was conspicuous at 6 weeks of age, and Purkinje cells were rarely present after 9–10 weeks of age (data not shown). To further characterize the spot-like structures, immunofluorescent labeling with SMI 31 (red) and nuclear counterstaining with DAPI (blue) was performed. The abnormally enlarged SMI 31-positive structures were devoid of nuclei (Fig. 1*G*). Occasional perikaryal staining was recognized by the presence of a nucleus within a larger-sized spot (Fig. 1*H*, *arrow*) relative to the more abundant smaller spot-like structures. Thus, the SMI 31-positive spot-like structures most likely represent the axonal spheroids described previously in human, murine, and feline NPC (Elleder et al., 1985; Higashi et al., 1993; Ong et al., 2001). These axonal spheroids often displayed a translucent core resembling lipid deposit (Fig. 1*I*, *arrowhead*). In general, neuronal somata swollen with lipid-like material were unstained (Fig. 1*I*, *arrow*). The SMI 32 antibody recognizing a nonphosphorylated epitope in 200 kDa high-molecular weight NF (NF-H) stained a few neuronal cell bodies in the brainstem and small fibers throughout the brain of +/+ mice (Fig. 1*J*). In −/− mice, SMI 32 immunolabeled numerous axonal spheroids (Fig. 1*K*, *arrows*) with a regional distribution (shown for the pons only, Fig. 1*K*) that was similar to those stained by SMI 31.

To verify that the histological abnormalities were a result of increased phosphorylation and/or accumulation of NFs, immunoblotting analyses were conducted with whole-brain lysates from +/+ and −/−, mice. Replicate blots were stained with SMI 31, SMI 32, and R39, a pan NF antibody recognizing all of the NF isoforms. The intensities of the NF-H, 160 kDa medium-molecular weight (NF-M), and 68 kDa low-molecular weight (NF-L) NF bands stained with R39 from four 8-week-old *npc-1* mice and four controls were quantitated densitometrically, and the results indicated that the levels of the NF-H, NF-M, and NF-L were increased by 2.4-, 1.5-, and 1.17-fold ($p < 0.03$ for all), respectively, in the *npc-1* mice. These changes in NF levels were observed, whereas the levels of many other proteins, such as tau, MAP2, cdk5 (shown in Figs. 4 and 5), and neuronal-specific nuclear protein (NeuN) (Brazelton et al., 2000), in the same samples, were invariant. The results for NeuN are shown in Figure 2 and indicate equal protein loading of all lanes. With respect to phosphorylation, a sixfold increase in SMI 31 immunoreactivity was observed with NF-M, but there was no significant increase in SMI31 immunoreactivity with NF-H (Fig. 2, SMI 31). These results suggest that NF-M is the only hyperphosphorylated NF isoform *npc-1* mouse brain. They also imply that phosphorylation of NF-H did not increase in proportion to the increase in total NF-H levels, which corresponds to a net accumulation of nonphosphorylated NF-H in the diseased mice. This interpretation was supported by the marked increase in SMI 32 immunoreactivity with NF-H in the *npc-2* mouse brain samples (Fig. 2, SMI 32) and the increased SMI 32 immunoreactivity with axonal spheroids (Fig. 1*K*). Neither SMI 31 nor SMI 32 stained any bands corresponding to tau, as has been observed in AD (Sternberger and Sternberger, 1983).

Hyperphosphorylated tau and MAP2 accumulate in *npc-1* mouse brain

To determine whether other cytoskeletal proteins normally localized in axons and dendrites (i.e., tau and MAP2) (Binder et al., 1986; Matus, 1990) are also modified, their phosphorylation status was examined in *npc-1* mice. A library of anti-tau antibodies [i.e., paired helical filament-1 (PHF-1), CP-13, CP-22, MC-6, MC-1, ALZ-50, and TG-5] raised against PHFs from AD brains (Table 1) was used. Overall, the pattern of staining obtained with the phosphotau antibodies was similar in that bundles of fibers were stained in +/+ mice (Fig. 3*A*, PHF-1, *D*, CP-22) and numerous axon spheroids were detected in −/− mice (Fig. 3*B*, PHF-1, *E*, CP-22). Classic NFTs were not observed with any of the anti-tau antibodies. Some perikarya of neurons (Fig. 3*C*, PHF-1) in the brainstem and basal ganglia was visible. Tau-positive axon spheroids were seen in the brainstem, basal ganglia, and white matter of −/− mice as early as 5 weeks of age and became more conspicuous over the following 7 weeks (shown only in 8-week-old mice) (Fig. 3*B,C*, PHF-1, *E*, CP-22). The pattern of staining with the CP-10 antibody recognizing phosphothreonine-231 in tau was somewhat different from that of the above phosphotau antibodies. CP-10 exhibited weak staining of neuronal cell bodies in +/+ mice (Fig. 3*F*), but in −/− mice there was a dramatic increase in the staining of neurons both in the cytoplasm and in the nucleus (Fig. 3*G*, *arrow*). Some neurons containing foamy material were conspicuously unstained (Fig. 3*G*, *), but others were stained (Fig. 3*G*, *arrowhead*). Curiously, the conformation- and sequence-dependent tau antibodies ALZ-50, MC-1, and

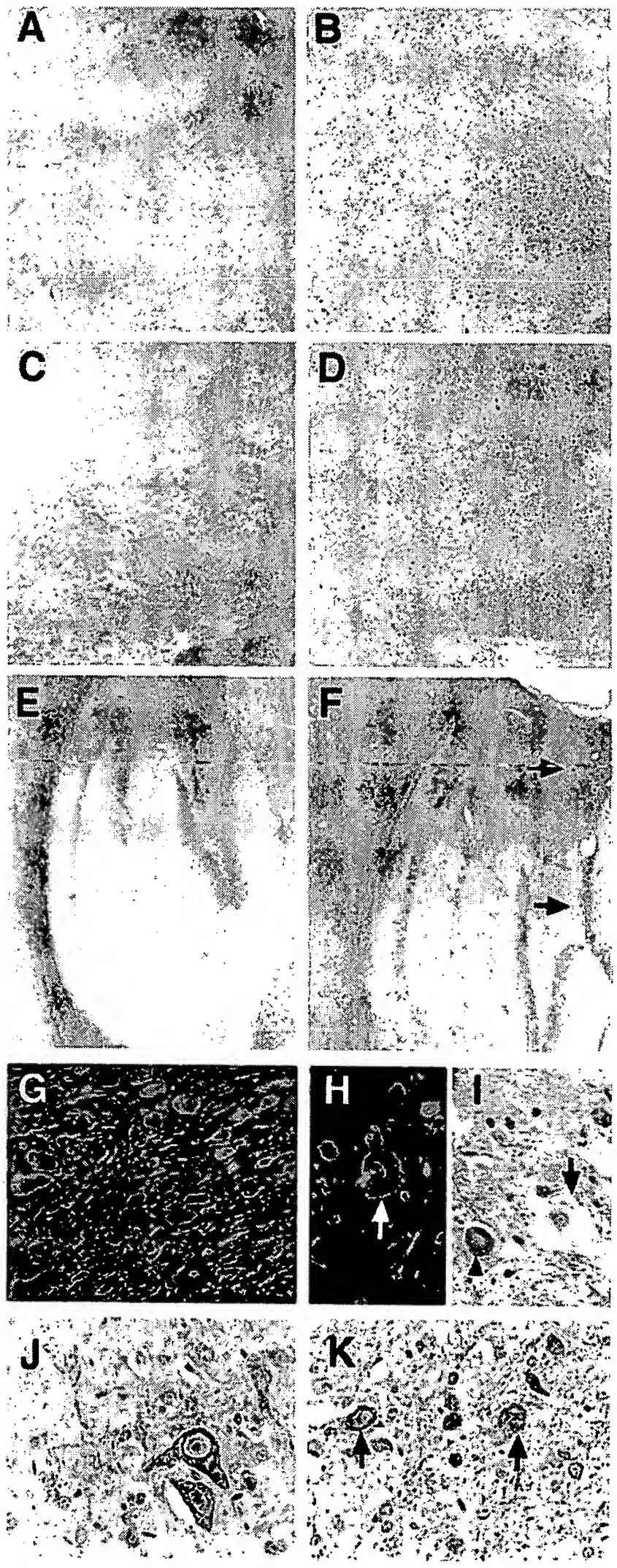

Figure 1. NF abnormalities in *npc-1* −/− mice. Immersion-fixed, paraffin-embedded sagittal brain sections from 7-week-old +/+ (*A*, *C*, *E*, *J*) and −/− (*B*, *D*, *F*, *G–I*, *K*) mice were immunolabeled with antibodies SMI 31 (*A–I*) or SMI 32 (*J*, *K*). For some sections, antibody binding was visualized with DAB (*brown*) and hematoxylin counterstain for highlight ing the nuclei of all cells (*blue-purple*) or with Cy-3 (*red*) and DAPI counterstain for nuclei (*blue*). In the +/+ mouse, SMI 31 positively stained bundles of processes throughout the brain [shown for the basal ganglia (*A*), brainstem (*C*), and hippocampus (*E*)]. In sharp contrast, intense spheroid-like structures were observed in vast numbers in the basal ganglia (*B*) and brainstem (*D*) and sparsely in the hippocampus (*F*, *arrows*). The absence of nuclei within the spheroidal structures (*G*) and their size suggest that they are cross sections of swollen axons. Some rarer, larger spots containing a nucleus resembled perikarya (*H*, *arrow* in cortex). The gigantically enlarged neurons containing storage were not stained with SMI 31 (*I*, *arrow*), in contrast to axonal staining with a translucent core (*I*, *arrowhead*). SMI 32 stained a few large neurons and small fibers in the pons of the +/+ mouse (*J*) and axonal spheroids principally in the brainstem of −/− mice (*K*, *arrows*). Magnification: *A–F*, 4×; *G–K*, 40×.

TG-5 did not react with axonal spheroids or any other pathological feature in the *npc-1* mice (data not shown). These antibodies stained axon fibers weakly in +/+ mice and fewer numbers of similar fibers in *npc-1* −/− mice (data not shown).

The AP-18 antibody recognizing a phosphoepitope in MAP2 gave positive results with perfusion-fixed sections only. In 7-week-old −/− mice, AP-18 displayed intense immunoreactivity in the somatodendritic compartment of large neurons of the thalamus (Fig. 3*I*, *arrows*). The apical dendrite was prominently labeled in many of these cells (Fig. 3*J*). Axonal spheroids in the thalamus and brainstem were labeled to a lesser degree with AP-18 (data not shown) than with tau or NF antibodies. The antibody did not show any staining in +/+ brain sections (Fig. 3*H*). AP-20, an antibody recognizing total MAP2 (i.e., the 240–280 kDa MAP2a and MAP2b isoforms), positively labeled the soma of many neurons throughout the brain, without any striking difference between the two groups of mice (data not shown).

Immunoblotting analyses of the microtubule-associated proteins was conducted with heat-stable supernatants from +/+ and −/− mice. An increase in tau phosphorylation was detected in *npc-1* −/− mice at the earliest time point tested (i.e., 4 weeks of age) and increased further through 10 weeks of age (only data from the 4- and 8-week-old mice are shown) (Fig. 4). In 4-week-old *npc-1* −/− mice, an increase in intensity of phosphotau antibody immunoreactivity with tau was seen compared with +/+ mice, although there was no striking change in electrophoretic mobility (Fig. 4, blots PHF-1, CP-13, and CP-10). In 8-week-old *npc-1* −/− mice, the triplet pattern of hyperphosphorylated tau similar to that of AD became apparent with most of the antibodies (Fig. 4, compare bands stained with CP-13 in 8-week-old *npc-1* −/− mice and AD). The average increase in immunoreactivity with phosphotau antibodies in 8-week-old *npc-1* mice compared with age-matched +/+ mice was threefold for CP-10 ($n = 4$) and PHF-1 ($n = 8$) and twofold for CP-13 ($n = 8$). The tau sequence and conformation antibodies TG-5, ALZ-50, and MC-1 showed no difference in the intensity of the tau bands between the two groups of mice, indicating similar levels of tau protein in both (Fig. 4, only TG-5 and ALZ-50 shown). Together, these data support a net increase in the phosphorylation of tau in *npc-1* mice.

Unexpectedly, CP-22 and MC-6, which were raised against purified PHF, did not react with tau in either +/+ or −/− mice. However, both antibodies recognized a protein of ~180 kDa that was markedly elevated in heat-stable supernatants from *npc-1* mice (Fig. 4, shown for CP-22 only). The size of this band is larger than the 97–110 kDa high-molecular weight tau species (Georgieff et al., 1991), and we found that it was more abundant

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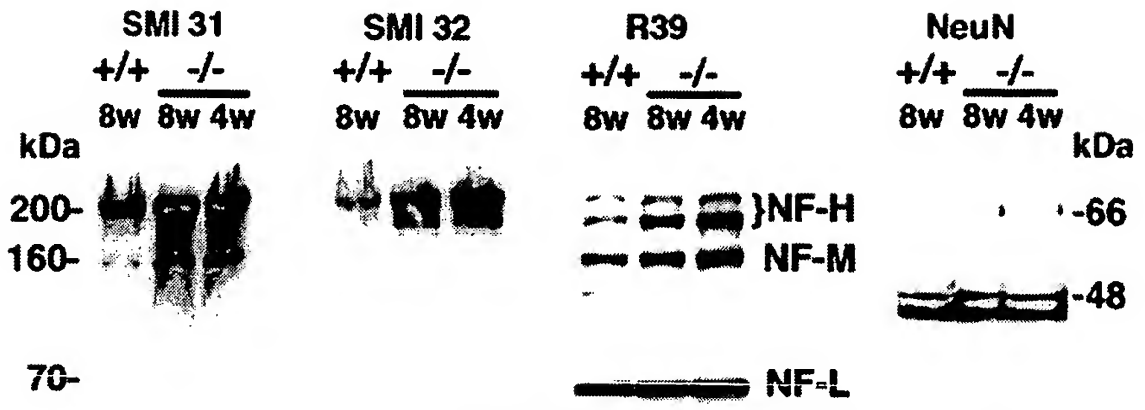

Figure 2. Increased levels of NF-H and increased phosphorylation of NF-M in *npc-1* −/− mice. Whole-brain lysates containing 10 μg of protein from 8-week-old +/+ and 4- and 8-week-old −/− mice were resolved on 8% gels, transferred to nitrocellulose, and blotted with the indicated antibodies. The pan NF antibody R39 identified the three major NF isoforms (marked NF-H, NF-M, and NF-L, respectively). The intensities of the bands visualized with SMI 31 and R39 were quantitated, and the results indicated a significant increase in the levels of all three isoforms and a marked increase in phosphorylation of the NF-M isoform. Equivalent loading of all samples is demonstrated with the NeuN antibody recognizing the 46–48 kDa neuronal-specific antigens.

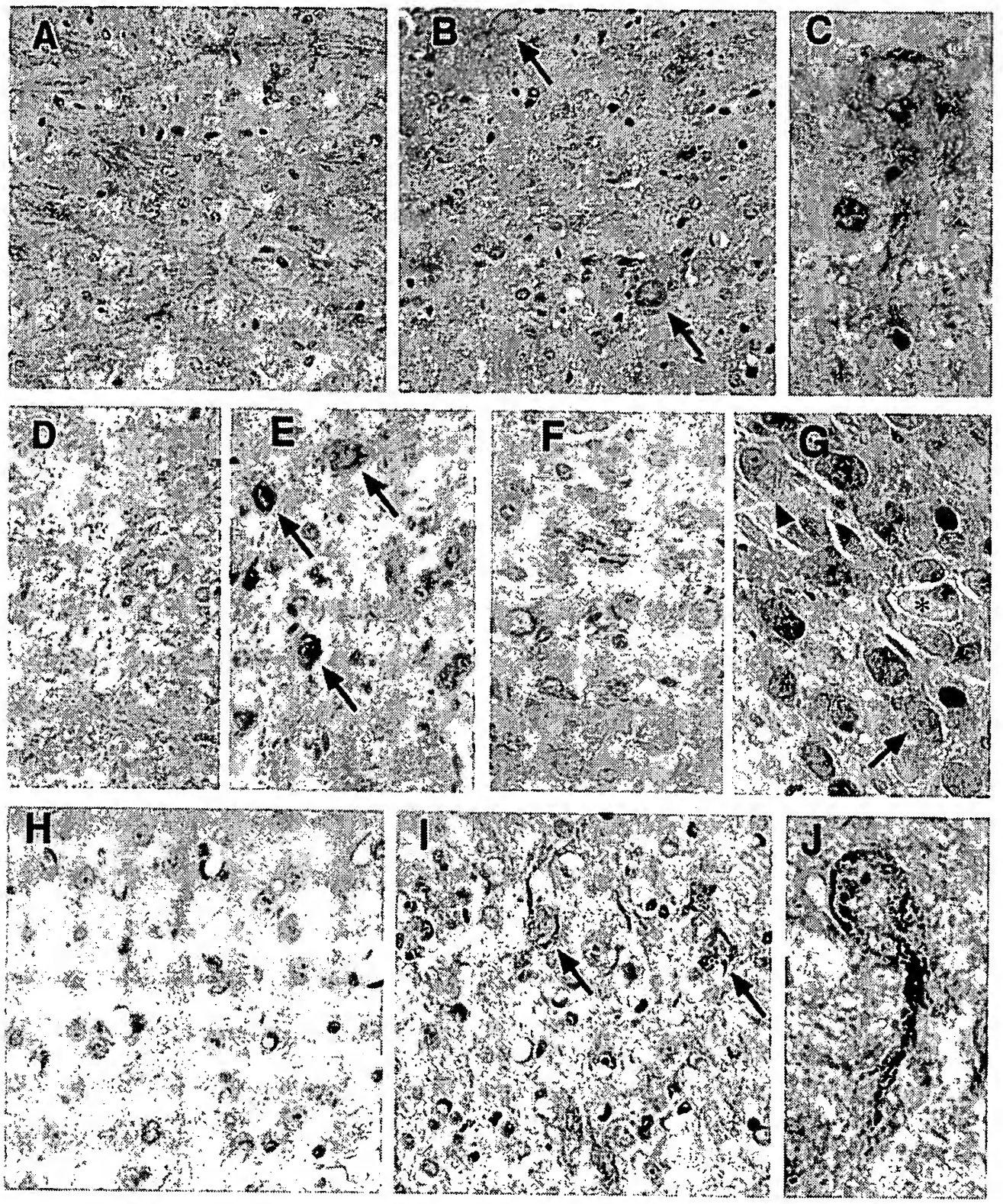

Figure 3. Tau and MAP2 abnormalities in the *npc-1* −/− mouse brain. Sagittal brain sections from +/+ (*A, D, F, H*) and 8-week-old −/− mice (*B, C, E, G, I, J*) were immunostained with PHF-1 (*A–C*), CP-22 (*D, E*), CP-10 (*F, G*), and AP-18 (*H–J*). PHF-1 stained bundles of processes in the brainstem and white matter of +/+ mice (*A*, pons) but numerous axonal spheroids (*B*, *arrows*) and some perikarya (*C*) of neurons in similar regions of the −/− mouse brain. CP-22 displayed much weaker staining of processes in +/+ mice (*D*) and a similar pattern of axonal spheroids (*arrows*) in the brainstem of −/− mice (*E*, pons). CP-10 exhibited weak staining of neuronal cell bodies in +/+ mice (*F*, pons), but in −/− mice there was a dramatic increase in the staining of neurons both in the cytoplasm and in the nucleus (*G*, *large arrow*). Some neurons containing foamy material were conspicuously unstained (*G*, *asterisk*), but others were CP-10 positive (*arrowhead*). AP-18 was primarily negative in the +/+ mouse brain (*H*) but labeled the soma and dendrites of several neurons (*arrows*) in the thalamus (*I*). *J*, One such neuron with prominent staining of the soma and apical dendrite. Magnification: *A, B, D–G, I, J*, 40×; *C, H*, 100×.

in the supernatant fraction without any heat treatment, suggesting that it is primarily heat labile (data not shown).

Consistent with the accumulation of phosphorylated MAP2 by immunohistochemistry, immunoblotting demonstrated that the phosphorylated high-molecular weight 240–280 kDa MAP2a and MAP2b isoforms of and the low-molecular weight 70 kDa MAP2c isoform were enriched in −/− mice, particularly at 4 weeks of age (Fig. 4, AP-18). At 8 weeks of age, no increase in phosphorylation of MAP2a and MAP2b was detected, but MAP2c appeared to be hyperphosphorylated. A duplicate blot

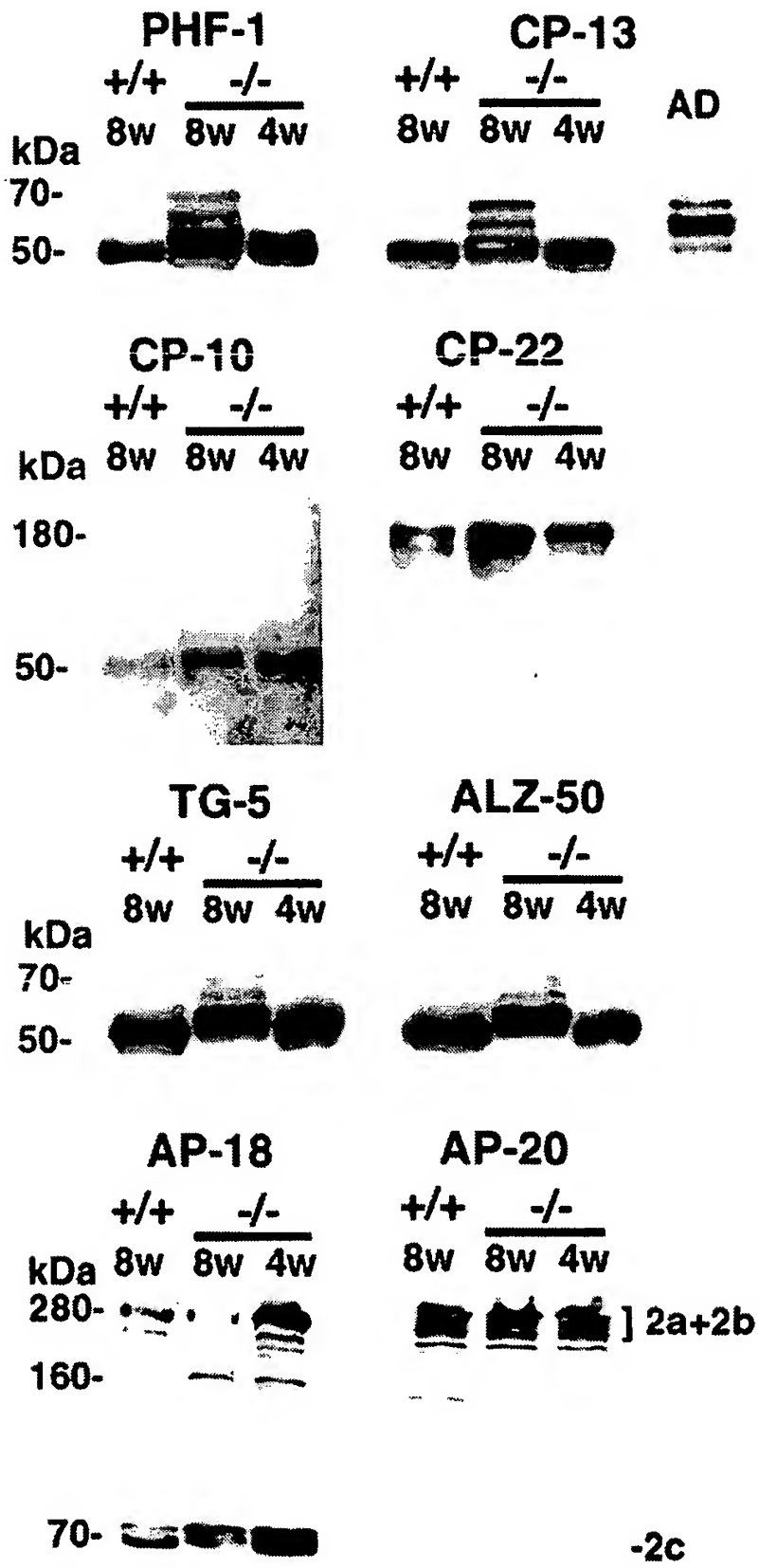

Figure 4. Increased phosphorylation of microtubule-associated proteins in *npc-1* −/− mice. Ten micrograms of whole-brain, heat-stable protein from +/+ and −/− mice and a typical AD case were blotted with the indicated antibodies (supernatant only for AP-18). Ten and 8% gels were used for detection of tau and MAP2, respectively. An increase in intensity and in the apparent molecular weight of tau was observed with PHF-1 and CP-13 in 4-week-old and even more so in 8-week-old −/− mice, consistent with hyperphosphorylation of tau. The pattern of tau bands at 8 weeks of age resembled that of AD-tau. CP-10-detected phosphorylated tau was increased threefold in −/− mice. CP-22 raised against and shown to recognize phosphorylated tau did not react with tau in the BALB/c strain of mouse. However, the antibody detected a molecule of ~180 kDa that was more highly phosphorylated in −/− mice. The tau sequence antibodies TG-5 and ALZ-50 displayed equivalent amounts of tau in all *lanes*. AP-18 immunoreactivity showed increased phosphorylation of the 280 kDa MAP2a and MAP2b and 70 kDa MAP2c protein −/− mice, particularly at 4 weeks of age. AP-20 did not recognize MAP2c but showed invariable amounts of total MAP2 (*2a+2b*) protein in all *lanes*.

stained with the AP-20 antibody recognizing the primary sequence in MAP2a and MAP2b showed similar amounts of these proteins in −/− mice relative to +/+ mice (Fig. 4, AP-20).

p25 levels and cdk5 activity are increased in *npc-1* −/− mouse brain

Previous evidence has suggested that the PHF-1, CP-13, SMI 31, and AP-18 phosphoepitopes are generated by cdk5 kinase (Berling et al., 1994; Patrick et al., 1999; Nguyen et al., 2001) that is enriched in axons (Tsai et al., 1993). Therefore, we compared the levels of cdk5 and its activator, p35, and cdk5 activity in −/− and +/+ mice. Immunoblotting analyses showed that the cdk5 levels were invariable in −/− mice compared with +/+ mice (Fig. 5*A*, cdk5, mouse). p35 levels were also unchanged, but an elevation in p25, a C-terminal truncated fragment of p35 (Patrick et al., 1999), was detected in −/− mouse brains from 5 to 12 weeks of age (Fig. 5*A*, p35 mouse). A similar pattern of constant cdk5 levels but increased p25 levels is seen in human NPC (Fig. 5*A*, human). Elevated p25 correlates with an increase in cdk5 activity, because p25 has a longer half-life and is a better activator of cdk5 than p35 (Patrick et al., 1999). To see whether cdk5 activity is altered in −/− mice, the kinase was immunoprecipitated from whole-brain lysates and tested for its phosphorylation activity toward histone H1 *in vitro* (Fig. 5*B*, cdk5 IP/kinase assay). The recovery of cdk5 in all of the immunoprecipitates was similar as judged by immunoblotting with a cdk5 monoclonal antibody (Fig. 5*B*, whole brain, cdk5 blots in *top row*), and Coomassie blue staining of the gel showed equivalent amounts of H1 substrate in the kinase reaction in all lanes (Fig. 5, whole brain, gel shown in *second row*). However, incorporation of labeled phosphate from [γ-^{32}P]ATP into H1 was significantly higher with cdk5 IPs from −/− mice than from +/+ mice (Fig. 5*B*, whole brain, H1 phosphorylation shown in *third row*). The increase was most striking between 4 and 7 weeks of age and less so at ≥8 weeks of age. The lower increase in cdk5 activity in older mice may be a result of neuronal loss, which at 8 weeks of age is most prominent in the cerebellum but becomes more significant in other regions as well in later stages of the disease. The average increase in cdk5 activity in whole-brain lysates from 5- to 10-week-old −/− mice was 1.6-fold (Fig. 5*B*, whole brain, histogram; $p < 0.01$; $n = 12$).

Given the regional distribution of axonal spheroids in *npc-1* brain, we also performed a regional comparison of cdk5 activities. The cerebellum, brainstem, and remaining forebrain from 5-week-old mice were analyzed. As was done with cdk5 IPs from whole-brain lysates, equivalent recovery of cdk5 in the IP and the equivalent amount of H1 were verified with the samples from different brain regions (Fig. 5*B*, *Forebr., B.stem, Cblm.*, respectively, cdk5 blot shown in *first row*, H1 protein in gel shown in *second row*). An increase in the amount of labeled phosphate incorporated into H1 was visible in the forebrain but was far more striking in the brainstem and barely noticeable in the cerebellum (Fig. 5*B*, *Forebr., B.stem, Cblm.*, H1 phosphorylation shown in *third row*). Quantitation of the data from two such determinations using samples from two different sets of mice revealed a sixfold increase in cdk5 activity in the brainstem and a twofold increase in the forebrain but no change in the cerebellum (Fig. 5*B*, histogram).

To support the significance of these cdk5 activity changes in NPC, another proline-directed kinase [i.e., glycogen synthase kinase-3β (GSK-3β)] that has been shown to phosphorylate sites similar to those of cdk5 in the above cytoskeletal proteins (Julien and Mushynski, 1998; Mattson, 2001) was explored. The GSK-3β

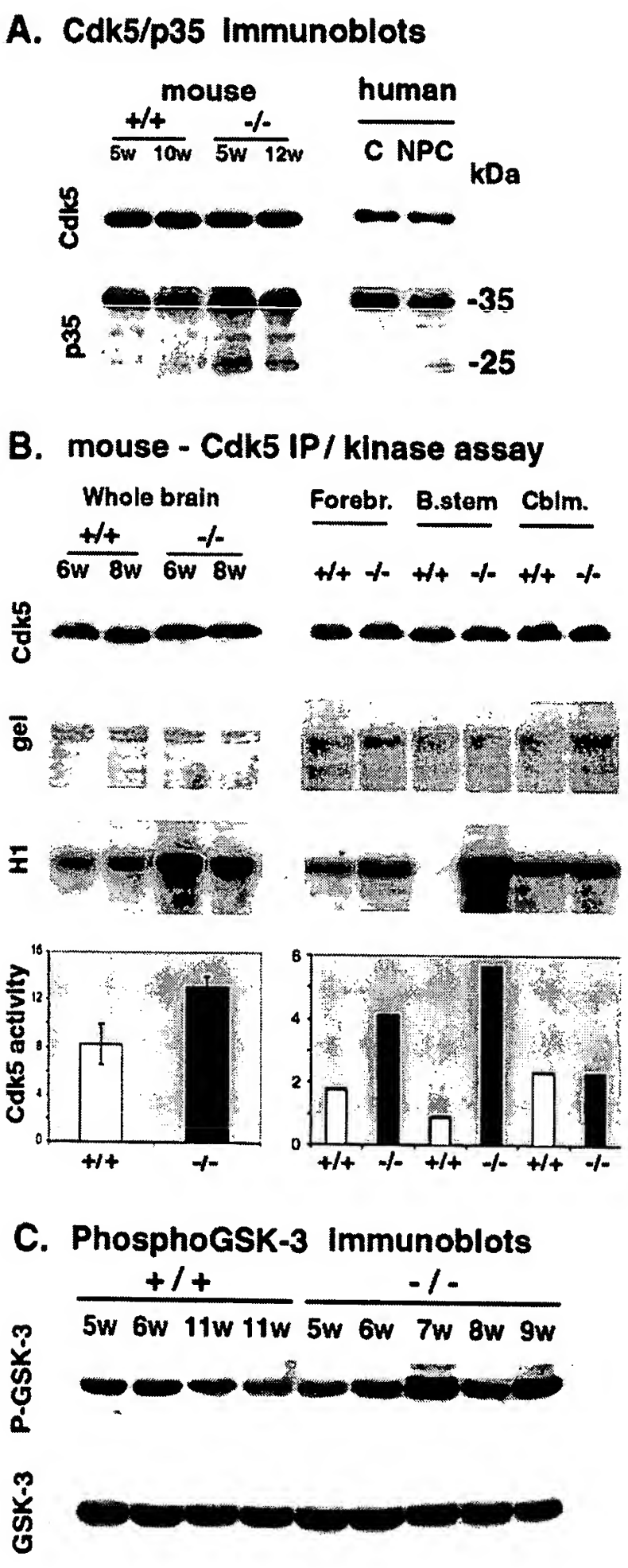

Figure 5. cdk5 and p25 abnormalities in the *npc-1* −/− mouse brain. *A*, cdk5/p35 immunoblots. Whole-brain lysates containing 40 μg of protein from +/+ and −/− mice were resolved on 12% gels and blotted with the indicated antibodies. cdk5 levels remained unchanged in −/− mice compared with controls. The p35 C-terminal antibody showed no difference in p35 levels but an enrichment of the 25 kDa p35 fragment (p25) in −/− mice (*left panel, mouse*). Similar preparations containing 100 μg of protein from a human control and an NPC hippocampus were immunoblotted with cdk5 and p35C antibodies. cdk5 and p35 levels were invariant, but p25 was elevated in NPC (*right panel, human*). *B*, cdk5 IP/kinase activity. Whole-brain lysates (*left panel*) from +/+ and −/− mice were immunoprecipitated with cdk5 (C-8) antibody. Immunoprecipitate (cdk5 IP) was immunoblotted with a different cdk5 antibody to show that the relative recoveries of enzyme in the samples were similar (*Cdk5*). Catalytic aliquots of cdk5 immunoprecipitate were incubated with kinase buffer containing histone H1 and [γ-^{32}P]-ATP for 20 min at 30°C, and the reaction mixtures were resolved on 12% gels. The incorporation of [γ-^{32}P]ATP into histone H1 (*H1*) was significantly higher with cdk5 IP from −/− mice compared with +/+ mice (*left panel*), on the basis of equivalent H1 loading demonstrated by staining of the gel with Coomassie blue (*gel*). The cdk5 activity in 5- to 10-week-old −/− mice was increased 1.5-fold over that seen in +/+ mice ($p < 0.01$; $n = 12$; *left panel*). *Right panel*, The increase in cdk5 activity in the forebrain (*Forebr.*) and brainstem (*B.stem*) but not in the cerebellum (*Cblm.*) of a 5-week-old −/− mouse compared with those of a 5-week-old +/+ mouse (all scales, ×1000). *C*, P-GSK-3β immunoblots. Whole-brain lysates containing 50 μg of protein from +/+ and −/− mice were resolved on 10% gels and blotted with P-GSK-3β antibody (*P-GSK-3*) or primary sequence-dependent antibody (*GSK-3*). The intensities of the resulting bands were quantitated densitometrically, and no significant differences ($p = 0.11$) were observed between the *npc-1* and control mice.

kinase is inactivated by phosphorylation of its ser9 residue (Grimes and Jope, 2001). The phospho-GSK-3β (P-GSK-3β) (ser9) antibody recognizing this phosphoepitope provides a convenient means of detecting changes in the activity of the kinase by tracing the phosphorylation status of this site. This P-GSK-3β antibody and another primary sequence-dependent GSK-3β antibody were used to explore the phosphorylation status and levels of GSK-3β in the *npc-1* mouse brain. Replicate blots were generated using brain lysates from five 5- to 9-week-old *npc-1* mice and three controls of similar ages. One blot was stained with the P-GSK-3β antibody and the other with the sequence antibody (GSK-3); neither antibody revealed any consistent change in GSK-3β immunoreactivity in NPC (Fig. 5*C*). This experiment was run in duplicate, and the intensities of the bands obtained with each antibody in the *npc-1* mice were compared with the corresponding bands in control mice. There was no significant difference ($p = 0.11$) in kinase levels or activity in the *npc-1* mice (Fig. 5*C*). Reprobing the blots with NeuN antibody confirmed that all of the lanes were equally loaded (data not shown).

p25 accumulates and colocalizes with hyperphosphorylated NFs in axon spheroids of NPC −/− mouse brain

To further determine whether the cdk5 activity changes were relevant to the cytoskeletal pathology in *npc-1* mice, immunohistochemical studies of cdk5 and p35 were conducted using perfusion-fixed brain sections. Despite the constant cdk5 levels in the immunoblots discussed above, cdk5 antibodies displayed an increase in immunoreactivity in some deformed neurons (Fig. 6*B*, *arrow*) and axon speroids (*arrowhead*) in the brainstem and basal ganglia of −/− mice compared with +/+ mice of the same age (Fig. 6*A*). An N-terminal p35 antibody recognizing only p35 but not p25 stained bundles of fibers in +/+ mice and fewer numbers of fibers in −/− mice but no axonal spheroids (data not shown). In contrast, a C-terminal p35 antibody recognizing both p35 and p25 exhibited intense and extensive staining of axonal spheroids in the brainstem, basal ganglia, and white matter of the cerebellum (Fig. 6, only brainstem shown, *F, green*), but in +/+ mice, its immunoreactivity was faint and localized in fibers and in soma of a few neurons (*arrows* in Fig. 6*C, green*). C-terminal p35 antibody-specific staining of NFTs suggested the accumulation of p25 in NFTs of the AD brain (Patrick et al., 1999). Thus, it appears that the C-terminal p35 antibody-specific staining of axonal spheroids

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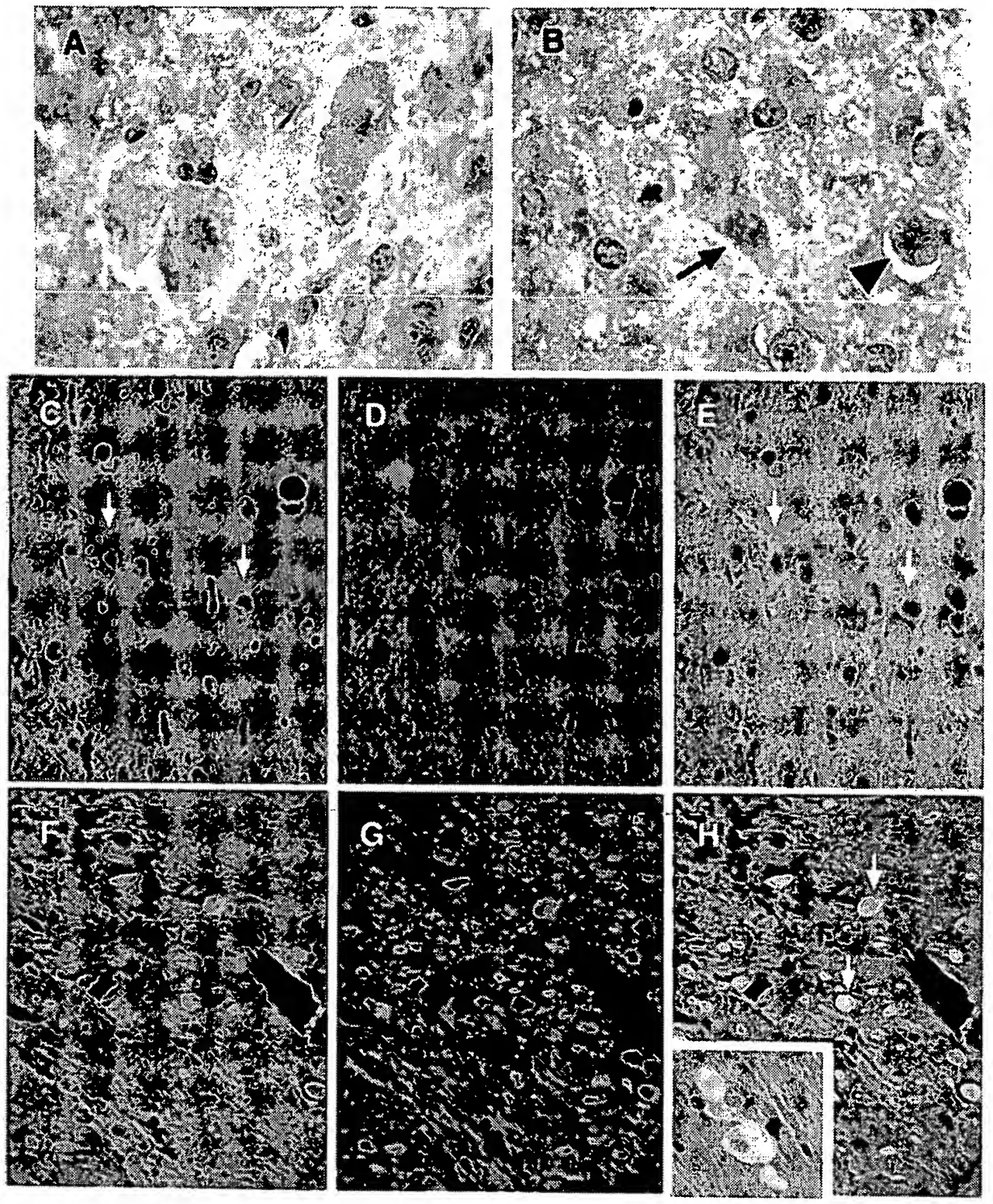

Figure 6. p35 accumulates and colocalizes with phosphorylated NF in axonal spheroids of *npc-1* −/− mice. Perfusion-fixed, paraffin-embedded sections from 7-week-old +/+ (*A*, *C–E*) and −/− (*B*, *F–H*) mice were immunohistochemically labeled with cdk5 (*brown*) and counterstained with hematoxylin (*blue-purple*) or immunofluorescently labeled with p35 (Alexa 488, *green*) and SMI 31 (Cy-3, *red*) and counterstained with DAPI (*blue*). cdk5 barely stained neurons in +/+ mice (*A*, pontine nucleus). In contrast, cdk5 immunoreactivity was increased in the soma of some deformed neurons (*arrow*) and in the axonal spheroids (*arrowhead*) in the brainstem of −/− mice (*B*, pontine nucleus). p35 faintly labeled the soma of neurons in the brainstem of +/+ mice (*arrows* in *C* and *E*, pons) and displayed little colocalization with SMI 31 immunoreactivity (*D* and *merged image* in *E*). In the −/− mouse brain, intense p35 immunoreactivity was seen in the axonal spheroids (*F*, pons), which perfectly matched the accumulation of SMI 31 immunoreactivity (*G* and *arrows* in *merged image* in *H*). *Inset* in *H*, Gigantically enlarged axons containing p35 (*green*) and SMI 31 (*red*) immunoreactivity in the cerebral cortex. Magnification: *A*, *B*, 100×; *C–H*, 40×.

may also suggest that increased p25 is localized to pathological structures in *npc-1* mouse brain. To gather additional support for this idea, double labeling with C-terminal p35 antibody (green) and SMI 31 (red) was performed. Wild-type sections did not show significant colocalization of C-terminal p35 antibody immunoreactivity and SMI 31 (Fig. 6*C,D*, respectively, and *merged image* in *E*). The micrograph shown from the brain of a 7-week-old *npc-1* −/− mouse instead indicates considerable overlap in localization of the two antigens in axonal spheroids (*arrows*, Fig. 6, C-terminal p35 antibody, *green*, *F* and *H*, SMI 31, *red*, *G* and *H*) throughout the brain.

DISCUSSION

The cardinal findings of this study include elevated levels of NF-H and hyperphosphorylation of NF-M, tau, and MAP2 in the *npc-1* mouse brain. These changes coincide with deregulation of the neuronal cdk5/p35 kinase complex. Accelerated proteolytic cleavage of p35 to p25, a more powerful cdk5 activator, correlates with increased cdk5 activity, and p25 colocalizes with hyperphosphorylated cytoskeletal proteins in the most conspicuous neuropathological lesion, the axonal spheroid. These focally enlarged axonal abnormalities are concentrated in the brainstem, basal ganglia, and white matter of the cerebellum, regions containing long myelinated axons and having high *NPC-1* gene expression levels (Prasad et al., 2000). Their abundance is directly proportional to the increase in cdk5 activity in each brain area. Chronologically, cdk5 activation and hyperphosphorylation are dramatic at 4 weeks of age, the earliest time point studied here, and axonal spheroids were first detected in the pons at 5 weeks of age. The severity of these changes escalated rapidly, reaching a maximum at 8–9 weeks of age, after which point marked brain atrophy became obvious. All of the biochemical changes detected here are observed in human NPC (Klünemann, Bu, Husseman, Elleder, Suzuki, Salamant, Love, Budka, Fligner, Bird, Jin, Nochlin, and Vincent, unpublished observations), but it is difficult to evaluate their temporal characteristics using autopsy brain tissue. The sequence of neuropathological events that we have delin-

eated in the *npc-1* mouse highlights an opportunity to inhibit the NPC neurodegenerative cascade with cdk5 inhibitors. Our studies also emphasize the value of the *npc-1* mouse model for testing and developing this therapeutic strategy and unraveling additional details of NPC neuropathogenesis.

NFTs were not detected in the *npc-1* mouse brain using tau antibodies or Bielchowsky silver reagent (data not shown), confirming previous studies that used thioflavin S (German et al., 2001a) and in contrast to the widespread occurrence of NFTs in human NPC (Auer et al., 1995; Suzuki et al., 1995). Curiously, even with a threefold increase in the phosphorylation of tau at some sites, the *npc-1* mouse brain was devoid of TG-5, ALZ-50, and MC-1 immunoreactivity. Such sequence- and conformation-dependent tau antibodies rarely stain neurons of normal rodent or human brain; their epitopes are sensitive to fixation, but they react strongly with AD neurons because their fixation sensitivity is alleviated by phosphorylation and aggregation (Pollock and Wood, 1988; Papasozomenos, 1989; Weaver et al., 2000). Our data would argue that hyperphosphorylation of tau at sites 202 (CP-13) and 396–404 (PHF-1) in axons and of CP-10-positive tau in neuronal soma are inadequate for eliciting TG-5, ALZ-50, and MC-1 immunoreactivity in either neuronal compartment. Perhaps phosphorylation at additional sites or other modifications (Gonzalez et al., 1998; Takeda et al., 2000) is required to expose these epitopes *in situ*. The absence of such additional modifications in the *npc-1* mouse brain might also be the key to their inability to form NFTs. The CP-22 and MC-6 antibodies recognize phosphothr-175 and phosphoser-235 in lysine–serine/threonine–proline motifs, respectively, in AD PHF (P. Davies, unpublished observations). Neither antibody reacted with tau in *npc-1* mice, suggesting that these sites are not phosphorylated in NPC. Instead, both antibodies recognized a 180 kDa heat labile protein present in axon spheroids. The possibility of this protein being a tau aggregate is excluded by its negative staining with other tau antibodies. It could be a degradation product of a larger protein containing similar proline-directed phosphorylation sites (i.e., NF-H or MAP2). All three MAP2 isoforms are hyperphosphorylated in the neuronal somatodendritic compartment of *npc-1* mice, but additional experiments are required to clarify their relationship with the 180 kDa protein.

Regarding the individual NF isoforms, the increase in NF-H levels was greater than the increase in SMI 31 immunoreactivity with NF-H; the increase in NF-M levels was less than the increased SMI 31 immunoreactivity with NF-M. These data translate into a selective increase in expression or reduced phosphorylation or degradation of NF-H and a selective increase in NF-M phosphorylation in NPC. The robust staining of axon spheroids with SMI 31 and SMI 32 suggests that a mixture of nonphosphorylated and hyperphosphorylated NF protein accumulates in the lesions. These results confirm earlier electron microscopic evidence for intermediate filaments in spheroids from human NPC (Elleder et al., 1985). However, axons normally contain hyperphosphorylated NFs, whereas perikarya contain hypophosphorylated NFs (Pant and Veeranna, 1995). Many neurodegenerative diseases, such as AD (Schmidt et al., 1991; Nixon, 1993), Parkinson's disease (Forno et al., 1986), and amyotrophic lateral sclerosis (ALS) (Julien, 1995), are characterized by perikaryal accumulations of hyperphosphorylated NFs. Axonal dilations have been documented in ALS and experimental models of axotomy (Stone et al., 2001) and in transgenic mice overexpressing four repeat human tau (Spittaels et al., 1999). These conditions are also associated with perikaryal NF or tau accumulation, implying disruption of proximal axonal transport. The absence of somatic accumulation of hyperphosphorylated tau and NFs in *npc-1* mice suggests a defect downstream of the proximal axon in NPC. Using silver staining (Patel et al., 1999) and Golgi impregnation (Zervas et al., 2001), it was noted that terminal fields of axons and dendrites are the earliest sites of degeneration in *npc-1* mice. Together, these data suggest that NPC neurodegeneration proceeds along a distal-to-proximal axis in long axons. The integrity of these axons has been thought to depend on the cycling of cholesterol between surrounding glial cells and the axons themselves (Xie et al., 1999), which may explain their special vulnerability to the lipid disturbances caused by *NPC-1* mutations.

In accordance with this more "distal" origin of NPC axonopathy is the enrichment of cdk5 in distal axonal segments (Nikolic et al., 1996). cdk5 normally mediates phosphorylation of NFs, tau, and MAP2 and their detachment from microtubules in axons (Matsushita et al., 1996; Wada et al., 1998). In *npc-1* mice, elevated p25 levels appear to be the trigger for cdk5 activation in distal axons, because p25 was enriched in cdk5 IPs from *npc-1* brain and colocalized with hyperphosphorylated NFs in axonal spheroids. Moreover, the levels and activity of the GSK-3β kinase, another enzyme that has been implicated in NF and tau phosphorylation, were unaltered in the *npc-1* mice. In transgenic mice overexpressing p25, a twofold increase in cdk5 activity resulted in tau and NF hyperphosphorylation and cytoskeletal pathology (Ahlijanian et al., 2000). Similarly, increased p25 activity and a twofold increase in cdk5 activity correlated with tau and NF hyperphosphorylation and motor neuron degeneration in the SODG37R ALS mouse model (Nguyen et al., 2001). In *npc-1* mice, we measured as much as a sixfold increase in p25-associated cdk5 activity in the brainstem, where hyperphosphorylation and cytoskeletal abnormalities were intense. A causal role for cdk5 in NPC is also supported by evidence linking its activity to phosphorylation of serines 202 and 396–404 in tau, the SMI 31 epitope in NF, and the AP-18 epitope in MAP2 (Berling et al., 1994; Patrick et al., 1999; Ahlijanian et al., 2000; Grant et al., 2001; Nguyen et al., 2001). Presently, it is not possible to exclude the involvement of other kinases in NPC. Sawamura et al. (2001) reported upregulation of MAP kinase, and we (Bu, Klünemann, Suzuki, Husseman, Bird, Jin, and Vincent, unpublished observations) have observed aberrant expression of cell division cycle kinase (cdc2) and cyclin B1 in the *npc-1* mouse cerebellum. Even with equivalent recovery of cdk5 in cdk5 IPs from the cerebellum and other brain regions, cdk5 activity and p25 were not elevated in the cerebellum, suggesting that other kinases mediate hyperphosphorylation in this region (Sawamura et al., 2001). The cerebellum has low p35 expression but is rich in p39 (Tang et al., 1995), which is degraded to a more effective activator, p29 (Patzke et al., 2002). We did not explore p39/p29 here, but if p29 was elevated in the *npc-1* cerebellum, an increase in cdk5 activity would have been detected using cdk5 IPs. p35 expression is induced by MAP kinase (Harada et al., 2001), which may explain the sustained p35 levels in *npc-1* mice that were observed even with its increased conversion to p25. The thr-231 tau epitope recognized by the CP-10 antibody when phosphorylated is a better acceptor site for cdc2 than cdk5. CP-10 immunoreactivity is produced abundantly in *npc-1* mice and is the only tau phosphoepitope that we detected in neuronal soma. Thus, although cdk5 may be the principal effector of axonal tau and NF in NPC, a somatic pool of tau may be modified by a different proline-directed kinase.

Cholesterol, glycosphingolipids, and glycosyl-phosphatidylino-

sitol-anchored proteins cluster in distinct cell membrane microdomains, called lipid rafts, and raft-enriched membrane invaginations called caveolas (Kurzchalia and Parton, 1999) constitute "signal transduction centers," coordinating extracellular signals with neuronal function and stability (Brown and London, 1998; Masserini et al., 1999). cdk5 mediates such a link in axons (Maccioni et al., 2001), and it is intriguing that p35 through its interaction with Rac GTPase (Nikolic et al., 1998); calpain, the protease that converts p35 to cytosolic p25 (Kulkarni et al., 1999; Bialkowska et al., 2000; Kusakawa et al., 2000; Lee et al., 2000); and a fraction of cellular *NPC-1* (Garver et al., 2000) are all present in caveolas. We propose that caveolas play a crucial role in NPC neuropathology. Alterations of the caveolar scaffold protein caveolin-1 and of annexin II in *npc-1* mice (Garver et al., 1997a,b) support this idea. The demyelination induced by *NPC-1* mutations (Elleder et al., 1985; German et al., 2002) may potentiate axonal pathology, because myelin regulates the levels and activity of calpain (Chakrabarti et al., 1990; Persson and Karlsson, 1991), the expression and phosphorylation of NFs (Starr et al., 1996; Gotow et al., 1999), and the density and stability of axonal microtubules (Sanchez et al., 2000; Kirkpatrick et al., 2001). It should be possible to unravel the temporal and spatial relationships of these events in the *npc-1* mouse model.

REFERENCES

Ahlijanian MK, Barrezueta NX, Williams RD, Jakowski A, Kowsz KP, McCarthy S, Coskran T, Carlo A, Seymour PA, Burkhardt JE, Nelson RB, McNeish JD (2000) Hyperphosphorylated tau and neurofilament and cytoskeletal disruptions in mice overexpressing human p25, an activator of cdk5. Proc Natl Acad Sci USA 97:2910–2915.

Auer IA, Schmidt ML, Lee VM, Curry B, Suzuki K, Shin RW, Pentchev PG, Carstea ED, Trojanowski JQ (1995) Paired helical filament tau (PHFtau) in Niemann-Pick type C disease is similar to PHFtau in Alzheimer's disease. Acta Neuropathol 90:547–551.

Berling B, Wille H, Roll B, Mandelkow EM, Garner C, Mandelkow E (1994) Phosphorylation of microtubule-associated proteins MAP2a,b and MAP2c at Ser136 by proline-directed kinases in vivo and in vitro. Eur J Cell Biol 64:120–130.

Bialkowska K, Kulkarni S, Du X, Goll DE, Saido TC, Fox JE (2000) Evidence that beta3 integrin-induced Rac activation involves the calpain-dependent formation of integrin clusters that are distinct from the focal complexes and focal adhesions that form as Rac and RhoA become active. J Cell Biol 151:685–696.

Binder LI, Frankfurter A, Rebhun LI (1986) Differential localization of MAP-2 and tau in mammalian neurons in situ. Ann NY Acad Sci 466:145–166.

Brazelton TR, Rossi FM, Keshet GI, Blau HM (2000) From marrow to brain: expression of neuronal phenotypes in adult mice. Science 290:1775–1779.

Brown DA, London E (1998) Functions of lipid rafts in biological membranes. Annu Rev Cell Dev Biol 14:111–136.

Camargo F, Erickson RP, Garver WS, Hossain GS, Carbone PN, Heidenreich RA, Blanchard J (2001) Cyclodextrins in the treatment of a mouse model of Niemann-Pick C disease. Life Sci 70:131–142.

Chakrabarti AK, Dasgupta S, Banik NL, Hogan EL (1990) Regulation of the calcium-activated neutral proteinase (CANP) of bovine brain by myelin lipids. Biochim Biophys Acta 1038:195–198.

Elleder M, Jirasek A, Smid F, Ledvinova J, Besley GT (1985) Niemann-Pick disease type C. Study on the nature of the cerebral storage process. Acta Neuropathol 66:325–336.

Erickson RP, Garver WS, Camargo F, Hossain GS, Heidenreich RA (2000) Pharmacological and genetic modifications of somatic cholesterol do not substantially alter the course of CNS disease in Niemann-Pick C mice. J Inherit Metab Dis 23:54–62.

Forno LS, Sternberger LA, Sternberger NH, Strefling AM, Swanson K, Eng LF (1986) Reaction of Lewy bodies with antibodies to phosphorylated and non-phosphorylated neurofilaments. Neurosci Lett 64:253–258.

Garver WS, Erickson RP, Wilson JM, Colton TL, Hossain GS, Kozloski MA, Heidenreich RA (1997a) Altered expression of caveolin-1 and increased cholesterol in detergent insoluble membrane fractions from liver in mice with Niemann-Pick disease type C. Biochim Biophys Acta 1361:272–280.

Garver WS, Hsu SC, Erickson RP, Greer WL, Byers DM, Heidenreich RA (1997b) Increased expression of caveolin-1 in heterozygous Niemann-Pick type II human fibroblasts. Biochem Biophys Res Commun 236:189–193.

Garver WS, Heidenreich RA, Erickson RP, Thomas MA, Wilson JM (2000) Localization of the murine Niemann-Pick C1 protein to two distinct intracellular compartments. J Lipid Res 41:673–687.

Georgieff IS, Liem RK, Mellado W, Nunez J, Shelanski ML (1991) High molecular weight tau: preferential localization in the peripheral nervous system. J Cell Sci 100:55–60.

German DC, Quintero EM, Liang CL, Ng B, Punia S, Xie C, Dietschy JM (2001) Selective neurodegeneration, without neurofibrillary tangles, in a mouse model of Niemann-Pick C disease. J Comp Neurol 433:415–425.

German DC, Liang CL, Song T, Yazdani U, Xie C, Dietschy JM (2002) Neurodegeneration in the Niemann-Pick C mouse: glial involvement. Neuroscience 109:437–450.

Gonzalez C, Farias G, Maccioni RB (1998) Modification of tau to an Alzheimer's type protein interferes with its interaction with microtubules. Cell Mol Biol (Noisy-le-grand) 44:1117–1127.

Gotow T, Leterrier JF, Ohsawa Y, Watanabe T, Isahara K, Shibata R, Ikenaka K, Uchiyama Y (1999) Abnormal expression of neurofilament proteins in dysmyelinating axons located in the central nervous system of jimpy mutant mice. Eur J Neurosci 11:3893–3903.

Grant P, Sharma P, Pant HC (2001) Cyclin-dependent protein kinase 5 (Cdk5) and the regulation of neurofilament metabolism. Eur J Biochem 268:1534–1546.

Grimes CA, Jope RS (2001) The multifaceted roles of glycogen synthase kinase 3beta in cellular signaling. Prog Neurobiol 65:391–426.

Harada T, Morooka T, Ogawa S, Nishida E (2001) ERK induces p35, a neuron-specific activator of Cdk5, through induction of Egr1. Nat Cell Biol 3:453–459.

Higashi Y, Murayama S, Pentchev PG, Suzuki K (1993) Cerebellar degeneration in the Niemann-Pick type C mouse. Acta Neuropathol 85:175–184.

Julien JP (1995) A role for neurofilaments in the pathogenesis of amyotrophic lateral sclerosis. Biochem Cell Biol 73:593–597.

Julien JP, Mushynski WE (1982) Multiple phosphorylation sites in mammalian neurofilament polypeptides. J Biol Chem 257:10467–10470.

Julien JP, Mushynski WE (1998) Neurofilaments in health and disease. Prog Nucleic Acid Res Mol Biol 61:1–23.

Kirkpatrick LL, Witt AS, Payne HR, Shine HD, Brady ST (2001) Changes in microtubule stability and density in myelin-deficient shiverer mouse CNS axons. J Neurosci 21:2288–2297.

Kulkarni S, Saido TC, Suzuki K, Fox JE (1999) Calpain mediates integrin-induced signaling at a point upstream of Rho family members. J Biol Chem 274:21265–21275.

Kurzchalia TV, Parton RG (1999) Membrane microdomains and caveolae. Curr Opin Cell Biol 11:424–431.

Kusakawa G, Saito T, Onuki R, Ishiguro K, Kishimoto T, Hisanaga S (2000) Calpain-dependent proteolytic cleavage of the p35 cyclin-dependent kinase 5 activator to p25. J Biol Chem 275:17166–17172.

Lee MS, Kwon YT, Li M, Peng J, Friedlander RM, Tsai LH (2000) Neurotoxicity induces cleavage of p35 to p25 by calpain. Nature 405:360–364.

Liu Y, Wu YP, Wada R, Neufeld EB, Mullin KA, Howard AC, Pentchev PG, Vanier MT, Suzuki K, Proia RL (2000) Alleviation of neuronal ganglioside storage does not improve the clinical course of the Niemann-Pick C disease mouse. Hum Mol Genet 9:1087–1092.

Loftus SK, Morris JA, Carstea ED, Gu JZ, Cummings C, Brown A, Ellison J, Ohno K, Rosenfeld MA, Tagle DA, Pentchev PG, Pavan WJ (1997) Murine model of Niemann-Pick C disease: mutation in a cholesterol homeostasis gene. Science 277:232–235.

Love S, Bridges LR, Case CP (1995) Neurofibrillary tangles in Niemann-Pick disease type C. Brain 118:119–129.

Maccioni RB, Otth C, Concha II, Munoz JP (2001) The protein kinase Cdk5. Structural aspects, roles in neurogenesis and involvement in Alzheimer's pathology. Eur J Biochem 268:1518–1527.

March PA, Thrall MA, Brown DE, Mitchell TW, Lowenthal AC, Walkley SU (1997) GABAergic neuroaxonal dystrophy and other cytopathological alterations in feline Niemann-Pick disease type C. Acta Neuropathol (Berl) 94:164–172.

Masserini M, Palestini P, Pitto M (1999) Glycolipid-enriched caveolae and caveolae-like domains in the nervous system. J Neurochem 73:1–11.

Matsushita M, Tomizawa K, Lu YF, Moriwaki A, Tokuda M, Itano T, Wang JH, Hatase O, Matsui H (1996) Distinct cellular compartment of cyclin-dependent kinase 5 (Cdk5) and neuron-specific Cdk5 activator protein (p35nck5a) in the developing rat cerebellum. Brain Res 734:319–322.

Mattson MP (2001) Neuronal death and GSK-3beta: a tau fetish? Trends Neurosci 24:255–256.

Matus A (1990) Microtubule-associated proteins and the determination of neuronal form. J Physiol (Lond) 84:134–137.

Millat G, Chikh K, Naureckiene S, Sleat DE, Fensom AH, Higaki K, Elleder M, Lobel P, Vanier MT (2001) Niemann-pick disease type c: spectrum of he1 mutations and genotype/phenotype correlations in the npc2 group. Am J Hum Genet 69:1013–1021.

Naureckiene S, Sleat DE, Lackland H, Fensom A, Vanier MT, Wattiaux R, Jadot M, Lobel P (2000) Identification of HE1 as the second gene of Niemann-Pick C disease. Science 290:2298–2301.
Nguyen MD, Lariviere RC, Julien JP (2001) Deregulation of Cdk5 in a mouse model of ALS: toxicity alleviated by perikaryal neurofilament inclusions. Neuron 30:135–147.
Nikolic M, Dudek H, Kwon YT, Ramos YF, Tsai LH (1996) The cdk5/p35 kinase is essential for neurite outgrowth during neuronal differentiation. Genes Dev 10:816–825.
Nikolic M, Chou MM, Lu W, Mayer BJ, Tsai LH (1998) The p35/Cdk5 kinase is a neuron-specific Rac effector that inhibits Pak1 activity. Nature 395:194–198.
Nixon RA (1993) The regulation of neurofilament protein dynamics by phosphorylation: clues to neurofibrillary pathobiology. Brain Pathol 3:29–38.
Ong WY, Kumar U, Switzer RC, Sidhu A, Suresh G, Hu CY, Patel SC (2001) Neurodegeneration in Niemann-Pick type C disease mice. Exp Brain Res 141:218–231.
Pant HC, Veeranna (1995) Neurofilament phosphorylation. Biochem Cell Biol 73:575–592.
Papasozomenos SC (1989) Tau protein immunoreactivity in dementia of the Alzheimer type: II. Electron microscopy and pathogenetic implications. Effects of fixation on the morphology of the Alzheimer's abnormal filaments. Lab Invest 60:375–389.
Patel SC, Suresh S, Kumar U, Hu CY, Cooney A, Blanchette-Mackie EJ, Neufeld EB, Patel RC, Brady RO, Patel YC, Pentchev PG, Ong WY (1999) Localization of Niemann-Pick C1 protein in astrocytes: implications for neuronal degeneration in Niemann-Pick type C disease. Proc Natl Acad Sci USA 96:1657–1662.
Patrick GN, Zukerberg L, Nikolic M, de la Monte S, Dikkes P, Tsai LH (1999) Conversion of p35 to p25 deregulates Cdk5 activity and promotes neurodegeneration. Nature 402:615–622.
Patterson MC, Di Bisceglie AM, Higgins JJ, Abel RB, Schiffmann R, Parker CC, Argoff CE, Grewal RP, Yu K, Pentchev PG, Brady RO, Barton NW (1993) The effect of cholesterol-lowering agents on hepatic and plasma cholesterol in Niemann-Pick disease type C. Neurology 43:61–64.
Patzke H, Tsai LH (2002) Calpain-mediated cleavage of the cyclin-dependent kinase 5 activator p39 to p29. J Biol Chem 277:8054–8060.
Persson H, Karlsson JO (1991) Calpain activity in a subcellular fraction enriched in partially degraded CNS myelin fragments compared with myelin. Neurosci Lett 130:81–84.
Pollock NJ, Wood JG (1988) Differential sensitivity of the microtubule-associated protein, tau, in Alzheimer's disease tissue to formalin fixation. J Histochem Cytochem 36:1117–1121.
Prasad A, Fischer WA, Maue RA, Henderson LP (2000) Regional and developmental expression of the Npc1 mRNA in the mouse brain. J Neurochem 75:1250–1257.
Sanchez I, Hassinger L, Sihag RK, Cleveland DW, Mohan P, Nixon RA (2000) Local control of neurofilament accumulation during radial growth of myelinating axons in vivo. Selective role of site-specific phosphorylation. J Cell Biol 151:1013–1024.
Sawamura N, Gong JS, Garver WS, Heidenreich RA, Ninomiya H, Ohno K, Yanagisawa K, Michikawa M (2001) Site-specific phosphorylation of tau accompanied by activation of mitogen-activated protein kinase (MAPK) in brains of Niemann-Pick type C mice. J Biol Chem 276:10314–10319.
Schmidt ML, Lee VM, Trojanowski JQ (1991) Comparative epitope analysis of neuronal cytoskeletal proteins in Alzheimer's disease senile plaque neurites and neuropil threads. Lab Invest 64:352–357.
Scriver CR, Beaudet AL, Sly WS, Valle D, Childs B, Kinzler KW, Vogelstein B (2001) The metabolic and molecular bases of inherited disease. New York: McGraw-Hill.
Spittaels K, Van den Haute C, Van Dorpe J, Bruynseels K, Vandezande K, Laenen I, Geerts H, Mercken M, Sciot R, Van Lommel A, Loos R, Van Leuven F (1999) Prominent axonopathy in the brain and spinal cord of transgenic mice overexpressing four-repeat human tau protein. Am J Pathol 155:2153–2165.
Starr R, Attema B, DeVries GH, Monteiro MJ (1996) Neurofilament phosphorylation is modulated by myelination. J Neurosci Res 44:328–337.
Sternberger LA, Sternberger NH (1983) Monoclonal antibodies distinguish phosphorylated and nonphosphorylated forms of neurofilaments in situ. Proc Natl Acad Sci USA 80:6126–6130.
Stone JR, Singleton RH, Povlishock JT (2001) Intra-axonal neurofilament compaction does not evoke local axonal swelling in all traumatically injured axons. Exp Neurol 172:320–331.
Suzuki K, Parker CC, Pentchev PG, Katz D, Ghetti B, D'Agostino AN, Carstea ED (1995) Neurofibrillary tangles in Niemann-Pick disease type C. Acta Neuropathol 89:227–238.
Takeda A, Smith MA, Avila J, Nunomura A, Siedlak SL, Zhu X, Perry G, Sayre LM (2000) In Alzheimer's disease, heme oxygenase is coincident with Alz50, an epitope of tau induced by 4-hydroxy-2-nonenal modification. J Neurochem 75:1234–1241.
Tanaka J, Nakamura H, Miyawaki S (1988) Cerebellar involvement in murine sphingomyelinosis: a new model of Niemann-Pick disease. J Neuropathol Exp Neurol 47:291–300.
Tang D, Yeung J, Lee KY, Matsushita M, Matsui H, Tomizawa K, Hatase O, Wang JH (1995) An isoform of the neuronal cyclin-dependent kinase 5 (Cdk5) activator. J Biol Chem 270:26897–26903.
Tsai LH, Takahashi T, Caviness Jr VS, Harlow E (1993) Activity and expression pattern of cyclin-dependent kinase 5 in the embryonic mouse nervous system. Development 119:1029–1040.
Vanier MT, Rodriguez-Lafrasse C, Rousson R, Duthel S, Harzer K, Pentchev PG, Revol A, Louisot P (1991a) Type C Niemann-Pick disease: biochemical aspects and phenotypic heterogeneity. Dev Neurosci 13:307–314.
Vanier MT, Rodriguez-Lafrasse C, Rousson R, Gazzah N, Juge MC, Pentchev PG, Revol A, Louisot P (1991b) Type C Niemann-Pick disease: spectrum of phenotypic variation in disruption of intracellular LDL-derived cholesterol processing. Biochim Biophys Acta 1096:328–337.
Vincent I, Jicha G, Rosado M, Dickson DW (1997) Aberrant expression of mitotic cdc2/cyclin B1 kinase in degenerating neurons of Alzheimer's disease brain. J Neurosci 17:3588–3598.
Vincent I, Zheng JH, Dickson DW, Kress Y, Davies P (1998) Mitotic phosphoepitopes precede paired helical filaments in Alzheimer's disease. Neurobiol Aging 19:287–296.
Wada Y, Ishiguro K, Itoh TJ, Uchida T, Hotani H, Saito T, Kishimoto T, Hisanaya S (1998) Microtubule-stimulated phosphorylation of tau at Ser 202 and Thr 205 by cdk5 decreases its microtubule nucleation activity. J Biochem 124:738–746.
Walkley SU (1995) Pyramidal neurons with ectopic dendrites in storage diseases exhibit increased GM2 ganglioside immunoreactivity. Neuroscience 68:1027–1035.
Weaver CL, Espinoza M, Kress Y, Davies P (2000) Conformational change as one of the earliest alterations of tau in Alzheimer's disease. Neurobiol Aging 21:719–727.
Xie C, Turley SD, Pentchev PG, Dietschy JM (1999) Cholesterol balance and metabolism in mice with loss of function of Niemann-Pick C protein. Am J Physiol 276:E336–E344.
Zervas M, Somers KL, Thrall MA, Walkley SU (2001) Critical role for glycosphingolipids in Niemann-Pick disease type C. Curr Biol 11:1283–1287.